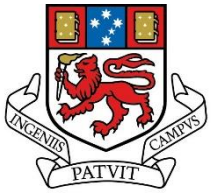

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Physiological mechanisms conferring ameliorative effects of nitric oxide and cytokinin on salinity stress tolerance in pea and barley

by

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Declaration of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and to the best of my knowledge contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

Shivam Sidana

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Abstract

In the current time, soil salinity is posing significant constraints in satisfying the needs of the regularly growing population by lessening the agricultural yield. Salinity is affecting nearly 7% of the world's land area leading to higher level economic loss due to decline in crop yield across the world. The approaching situation of food shortfall puts extreme pressure on devising new techniques to make agriculture productive in saline areas. One of the methods to counteract this problem is by developing salt tolerant plants either by genetic manipulation or by selective breeding. However, exogenous application of various organic products including phytohormones and inorganic chemicals to plants has shown the ameliorating effect on growth and productivity under different types of stress. Nitric oxide (NO) is one of the most important signalling molecules investigated in the last two decades, which played a significant role in plant growth under biotic and abiotic stress. Application of NO donor -sodium nitroprusside (SNP) significantly raised Na^+ efflux and compartmentalization in vacuoles maintaining intracellular ionic homeostasis in the leaves, whereas use of NO scavenger reversed the effects of SNP on the Na^+ / K^+ ratio. Taking into account, previous studies reporting the positive influence of NO on salt stressed plants, a comprehensive study of monitoring effect of NO foliar spray on the glasshouse grown barley and pea was undertaken. Pea (cv Onslow) and barley (cv Gairdner and CM 72) differed in their tolerance to salinity. After optimization, NO concentration (100 µM) was applied as a foliar spray on the plants of barley and pea. Results showed that NO application was unaffected in improving pea and barley growth under salt stress. It's reflected in the growth, CO_2 assimilation, chlorophyll content and photochemical efficiency of these plants under salt stress. The exogenous application of NO is mainly challenged by its 1) photo-instability 2) uptake 3) dose-time dependency factors.

Plant hormones have also been reported to elicit an adaptive response in plants under stress. The application of cytokinin (CYT) with different concentrations (20, 50 µM) has been investigated to see its effect on the salinity tolerance in this study. The amelioration effect of CYT was found to be dose dependent. The pre-treated pea plants with CYT were able to maintain high K^+/ Na^+ ratio which is vital for survival under salinity. CYT pre-treatment enhanced H^+-ATPase activity under salt stress which helped in reducing membrane depolarisation and reduced the K^+ efflux via depolarization-activated potassium outward rectifying (KOR) channels. Na^+ exclusion ability of pea mesophyll increased significantly ($P<0.05$) in CYT (20 µM) pre-treated samples compared with that of non-treated inducing

SOS1 in pea mesophyll leading to the extrusion of Na^+ from the cytosol. The management of oxidative stress by CYT was investigated by using non-invasive microelectrode ion flux measuring (MIFE) system. The results indicated the positive influence of CYT on the biomass yield, photosynthetic mechanism; ions flux response ($P<0.05$) though the results of mitigation of oxidative stress were not consistent. The experiments with optimized dose of CYT (5 µM) were repeated with barley (Gairdner) roots. CYT was able to elicit positive effect on root biomass and maintaining high K^+/ Na^+ ratio by preventing NaCl-induced K^+ loss from the roots and Na^+ accumulation. CYT treatment enhanced the SOS1-like activity and shifted the membrane potential of salt-treated root cells to more negative under saline conditions. CYT makes the plant salt tolerant by retaining K^+ which can be facilitated by better control of GORK (activated by depolarization) and good control of ROS-activated channels (such as NSCC). Pharmacological experiments showed that TEA^+ (voltage-gated K^+ channel blocker) effect was major while Gd^{3+} (NSCC blocker) effect was minor. CYT improves salinity stress tolerance via two concurrent mechanisms: (1) enhanced Na^+ efflux capacity originating form activation of SOS1-like Na^+/H^+ exchangers and (2) improved K^+ retention resulting from CYT activation of H^+-ATPase and better voltage control, preventing GORK-mediated stress-induced K^+ loss. There is also a potential evidence for CYT being able to desensitize GORK responses to ROS.

Abbreviations

ABA	Abscisic acid
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate
BSM	Basic Salt Media solution (1 mM NaCl; 0.5 mMKCl; 0.1 mM $CaCl_2$; pH 5.7 non-buffered)
CYT	Cytokinin
DEZ	Distal elongation zone
Fv/Fm	Maximal quantum efficiency of PSII
GORK	Guard cell K^+ outward-rectifier
HKT	High affinity K^+ transporter
KIR	K^+ inward-rectifying channel
KOR	K^+ outward-rectifying channel
LIX	Liquid ion exchanger
MIFE	Microelectrode ion flux measurement
MP	Membrane potential
MZ	Mature zone
NHX1	Na^+/H^+ exchanger 1
NO	Nitric oxide
NSCC	Non-selective cation channel
PCD	Programmed cell death
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
SOS	Salt overlay sensitive

SPAD	Soil-Plant Analyses Development

Table of Contents

Chapter 1: General introduction

Salinity as an issue

Soil salinity has become a severe environmental problem in many parts of the world. Globally, nearly 20% of the land is under cultivation, and in the case of irrigated land, about 33% is affected due to high salinity (Munns 2005). According to Food and Agricultural Organization (FAO) in 2000, the overall worldwide area of salt-affected soils (saline and sodic soils) encompasses 831 million hectares. It has been estimated that global salt induced land degradation and resulting production losses in irrigated areas could be as high as US$27.3 billion per year (Qadir et al., 2014). In Australia, salt is a natural constituent of the landscape (Nielsen et al. 2003); Therefore, the dominance of sodium salts has an impact on nearly 30% of the land area in Australia (Rengasamy 2002). Along with this environmental problem, there is a dire need to double the agricultural production to meet the needs of expectant fifty percent rise in global population by 2050 (Flowers 2004; Tester & Langridge 2010). This approaching situation puts extreme pressure on devising new techniques to make agriculture productive in saline areas.

Salinity poses several constraints on the growth and development of plants by influencing vital metabolic processes. The uptake of the water by the root is affected due to reduced water potential because of increased ionic concentration in the soil around roots (Munns and Termaat 1986). The turgor pressure in the cell decreases under salinity, thus affecting the cell elongation and ultimately growth. The water supply to the shoot is decreased which reduces transpiration rate and is a reason for stomatal closure (Brugnoli and Lauteri 1991). Moreover, uncontrolled salt entry into the plants increases salt concentrations inside the plant causing toxic effects (Ashraf and Khanum 1997). An increase in the concentration of Na^+ disturbs K^+ uptake through competitive inhibition (Nitsos and Evans 1969; Hall and Flowers 1973). Salinity also causes overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ion (O^{2-}), and hydroxyl radical ($\cdot OH$) (Hasegawa et al., 2000), thus leading to DNA, RNA, proteins, enzymes, lipids damage and eventual cell death (Apel & Hirt 2004). Salt-tolerant cultivars of barley maintained both low ROS-induced K^+ efflux (Cuin and Shabala, 2005 & 2007). The investigation conducted by the same group found that the application of compatible solutes (betaine and proline) are effective

in decreasing the K^+ efflux under salinity and oxidative stress. Though the accumulation of most of osmolytes in cytosol is non-toxic, but are energetically more expensive.

Salt tolerant plants respond to salinity by (a) decreasing Na^+ entry and accumulation of Na^+ inside cells, (b) enhancing Na^+ removal from shoot, intracellular compartmentalization or distribution to matured leaves, (c) maintaining of high K^+/Na^+ ratio inside the cytosol, (d) synthesizing, accumulating organic osmolytes and enhancing enzymatic as well as non-enzymatic antioxidant defense systems (Hasegawa et al. 2000; Tester and Davenport 2003).

Salt stress tolerance in plants can be improved by several means including genetic manipulations, seed pre-treatments and foliar sprays with agrochemicals (Krishnamurthy 1991; Flowers 2004). Salt tolerant genes could be introduced into the crop species through genetic manipulation, and this requires a thorough understanding of mechanisms of salt stress tolerance, the target sites/ defensive genes of salt stress, and signaling pathways in plants. The salt stress tolerance in plants is considered a multigenic trait (Lin et al. 2004; Yamaguchi and Blumwald 2005) which adds to the complexity in understanding the molecular and physiological mechanisms. Recent study by (Basil and Blumwald, 2014) that under normal growth conditions NHXs are critical regulators of K+ and pH homeostasis and have important roles, depending on their cellular localization, in the generation of turgor as well as in vesicular trafficking. (Basil and Blumwald, 2014) highlight novel and exciting functions of intracellular NHXs in growth and development, stress adaptation and osmotic adjustment. Intracellular proteins NHX1 and NHX2 are the two major tonoplast-localized NHX isoforms. NHX1 and NHX2 have similar expression patterns and identical biochemical activity. Reverse genetics showed functional redundancy of NHX1 and NHX2 genes. Growth of the double mutant nhx1 nhx2 was severely impaired, and plants were extremely sensitive to salinity stress (Barragan et al., 2012). So, genetic manipulation via transgenic has its limits and salt stress tolerance in plants is a multigenic trait due to which success has been rather scarce so far. Often claims have been made that through genetic manipulation of few genes salt stress tolerance in plants can be enhanced (Flowers 2004). Indeed, plant breeders have utilized genetic variability exits at intra and interspecific level to develop salt-tolerant crop plants, few examples of salt tolerant crops produced by conventional breeding are CSR10, CSR13, CSR27 rice cultivars (Mishra et al. 2003). However, reproductive barrier and low variation in gene pools for salt tolerance in most crops are the main limitations for genetic engineering (Ashraf et al. 2008). On the other hand, exogenous application of various organic and inorganic chemicals to plants has shown

to ameliorate salt stress in diverse plant species (Farooq et al. 2009). However, the molecular mechanisms behind these phenomena are poorly understood. The aim of my thesis is to address the role of nitric oxide (NO) and cytokinin (CYT) in imparting salt stress tolerance in plants.

The role of nitric oxide during salt stress

The nitric oxide (NO) is one of the signaling molecules that have been investigated in the last two decades for its role in biotic/abiotic stress tolerance in plants (Molassiotis et al. 2010; Hamayun et al. 2010). Foliar application of NO on soybean (Zhang et al. 2004), rice (Farooq et al. 2009) and maize (Wu et al. 2011) have shown to increase plant growth under salt stress conditions. The up-regulation of the antioxidant enzyme activities is considered as one of the reasons for the ameliorative effect of NO during salt stress. Indeed, the activities of ROS scavenging catalase, peroxidase, and superoxide dismutase enzymes have been much higher after NO donor sodium nitroprusside (SNP) spray (Guo et al. 2005). Further, NO donor application was found to up-regulate the expression of Arabidopsis inward K^+ channel (AKT1) in the roots of NaCl-treated *Kandelia. Obovata*, suggesting the significance of NO in increasing K^+ uptake in salt-treated plants. NO has also been found to reversibly inhibit the K^+ outward channel that facilitates salt-induced K^+ efflux (Chen et al. 2013) implying NO may participate in K^+ retention inside the cytosol during salt stress. The application of SNP significantly brought the Na^+ concentrations down in shoots and roots of *K. virginica* seedlings under salt stress; This suggests NO might have either prevented Na^+ entry or promoted Na^+ efflux out of the root tissue (Guo et al. 2009). Zhao et al. 2004signifies NO's involvement in the Na^+ sequestration. From above, it is evident that at the cellular level, NO application plays a pivotal role in modulating several salt tolerance mechanisms. However, limited information is available on how NO application could alter photosynthesis and ion homeostasis during prolonged salt exposure. The chapter three of my thesis addresses this knowledge gap by investigating the efficacy of NO as a foliar spray on glass house has grown two cultivars of barley (cv Gairdner & CM72) and pea (cv Onslow), in negating the adverse effect of salt stress.

The role of cytokinin during salt stress

Soil salinity elicits its negative effects by altering the hormonal ratio in root and shoot; under salinity abscisic acid level increases and that of cytokinin (CYT) and gibberellins decreases (Zhang and Zhang 1994). As cytokinin plays an essential role in leaf senescence,

stomatal opening, shoot morphogenesis, and cell division (Mok, 1994), any alteration in cytokinin levels during salt stress will have a profound effect on these processes. However, the effect of CYT in amelioration of salinity in plants is ambiguous as some reports present evidence in favor of CYT and others against it. No significant effect of kinetin was observed in guard cell inward-rectifying K^+ channel (KAT1) (Mori et al. 2000). Exogenous application of kinetin stimulated K^+ influx in roots hair cells by regulating K^+ permeable channels present in the root epidermis (Schumaker and Gizinski, 1993). However, there is no information on which specific K^+ transport mechanisms are modulated by CYT. The CYT also has a role in the management of oxidative stress because Arabidopsis CYT-deficient mutants *ipt1, ipt3, ipt5, ipt7* have hampered in ROS scavenging (Nishiyama et al., 2012) and enhancement in the activities of antioxidative enzymes ascorbate peroxidase (APX) and catalase (CAT) in dark-induced senescence on exogenous applications of CYT has been reported (Zavaleta-Mancera et al. 2007). However, there is no information on how CYT could modulate salt-induced oxidative stress. The chapter four and five in this thesis are an effort to fill the aforementioned knowledge gap by investigating the effect of CYT on ionic (Na^+/K^+ ratio) and ROS homeostasis in plants.

The literature review in this thesis is a compilation of detrimental effects of salinity on plant and mechanisms adopted by them to cope with it. It also discusses the role of NO and CYT in the growth and developmental processes in plants and their effect in imparting salt tolerance. The third chapter studies the combined effect of salt stress and foliar application of NO on the two cultivars of barley (cv CM72 & Gairdner) and pea (cv Onslow). The impact is evaluated through measurement of chlorophyll content and photochemical efficiency of the leaves under *in vitro* conditions and the same parameters along with biomass accumulation, ions flux response and CO_2 assimilation under *in vivo* conditions. The fourth chapter explains the effect of CYT on the biomass yield and photosynthetic mechanism. Microelectrode ion flux measurement (MIFE) is employed to monitor ions flux response & mitigation of oxidative stress in pea under NaCl treatment. In fifth chapter capability of CYT to alleviate the adverse effects of water deficit and salinity on barley roots is addressed by monitoring membrane potential, different ions (K^+, Ca^{2+}, Na^+ & H^+) flux responses, management of ROS generation employing MIFE. Also, pharmacological experiments are conducted to study the impact of CYT on the K^+ efflux by influencing the K^+ channels. The sixth chapter discusses the results of the experiments complied in the thesis and summarizes conclusions drawn and scope for future work.

Chapter 2: Literature review

2.1. Detrimental effects of salinity in plants

Salinity suppresses plant growth and productivity by inflicting both ionic and osmotic stresses (Munns and Tester 2008). Increased concentration of salts in the soil makes roots incapable of drawing water due to reduced water potential. Consequently, the build-up of salt concentrations inside plant hampers several metabolic activities leading to toxicity (Tavakkoli et al., 2011). The loss of turgor pressure in mesophyll leads to closure of stomata, which is the main implication of osmotic stress. The osmotic constraints of salinity get manifested when accumulated salts inside the plants reach lethal concentrations (Munns and Tester 2008). The accumulation of Na^+ ions inside plant cells leads to altered K^+ uptake and impaired metabolic processes (Blumwald 2000). In addition to ionic and osmotic components, salt stress, like other abiotic stresses, causes oxidative stress through an increase in reactive oxygen species (ROS) production. Plant adaptations to salinity consist of three distinct types; (1) osmotic stress tolerance, (2) ion exclusion, and (3) tissue tolerance to accumulated ions (Munns and Tester 2008).

2.1.1 Osmotic and ionic stress

The uptake of the water by the root is affected by reduced water potential resulting from increased ionic concentration in the soil around roots. The turgor pressure in cells drops under salinity, thus affecting the cell elongation and ultimately growth (Aroca et al., 2012). The water supply to shoot is decreased, and that is the reason for stomatal closure (Brugnoli and Lauteri 1991). The closure of the stomata limits photosynthesis by cutting down the availability of CO_2. Reduced photosynthesis sets other detrimental processes such as photorespiration and ROS generation (Kuvykin et al., 2011).

The toxicity posed by ions during salt stress has been better researched in plants than the effect of osmotic stress (Ashraf and Khanum 1997; Tester and Davenport 2003). Toxicity is due to increased concentration of cytosolic Na^+ as it disrupts the activities of the cytosolic enzymes (Hall and Flowers 1973; Benito et al. 2014). Salinity leads to a decrease in K^+ content (Wu et al., 2013) and an increase in ionic strength within the cells, causing denaturation

of proteins (Kronzucker et al., 2013). Much of the significance has been given to K^+/Na^+ ratio within the cytosol in imparting salt tolerance in plants. However, K^+/Na^+ ratio measured in the whole tissue using current methods are not correct. The methods used did not consider the contribution of vacuolar compartmentalization of Na^+ to combat salinity (Shabala and Mackay 2011). The technical difficulties associated with the determination of K^+/Na^+ ratio in the cytosol means an absence of threshold values for K^+/Na^+ ratio during salinity damage in plants. The upper safe concentration of Na^+ in the cytosol is not clear.

2.1.2 Oxidative stress

Oxidative stress is a primary component of most abiotic stresses (Apel and Hirt, 2004), and is also one of the restrictive factors of plant growth (Mittler, 2006). The oxidative stress takes place when the rate at which ROS is produced surpass the ability of detoxification by antioxidant systems leading to damage of biomolecules (DNA, RNA, proteins, enzymes, lipids) resulting in cellular dysfunction, and eventually cell death (Halliwell, 2006). Chloroplasts, peroxisomes, cell wall NADPH-oxidase, and mitochondria are the sites of ROS generation (Mittler, 2002; Bose et al., 2014). Plants have adapted two mechanisms to prevent ROS damage either by preventing ROS generation or by increasing their scavenging.

2.1.3 Disturbance to K^+ and Ca^{2+} homeostasis

The maintenance of the intracellular K^+ homeostasis is critical for turgor pressure, cytosolic pH homeostasis, enzyme activation, loading of photoassimilates into are phloem, and photosynthesis to name a few (Shabala 2003; Dreyer and Uozum 2011; Shabala and Pottosin, 2014). Potassium also plays a role in controlling apoptosis in animal cells (Hughes and Cidlowski 1999). Under salinity stress, the net K^+ uptake by the plants is impaired leading to K^+ deficiency inside the cytosol, an increase in oxidative stress, and eventually programmed cell death in plants. The efficiency to control K^+ efflux across the plasma membrane imparts tolerance to a range of abiotic stress conditions including salt stress (Shabala et al., 2007; Demidchik et al., 2010).

The concentration of cytosolic calcium is in nano molar range, while it is in the range of 1- 10 mM in endoplasmic reticulum and vacuole under normal conditions (Rudd and Franklin Tong, 2001). Under salt stress, a sudden increase in the concentration of the cytosolic

calcium is observed, which is toxic to plants (Tracy et al., 2008). To get rid of the excess cytosolic calcium, plants either store it into apoplast or the vacuoles or endoplasmic reticulum. However, some studies had reported reduced cytosolic calcium in root tissue, or root protoplasts when plants were treated with salt (Lynch et al., 1987). This variation in results arose because the different studies employed different techniques (cyto histochemical techniques or flame photometery) and plant materials (whole plant or specific cells) in several studies.

2.2. Salt tolerance mechanisms in plants

Salt tolerance is the ability of plants to grow and complete their life cycle in the environment with high soluble salt (≈ 200mM) concentrations (Flower et al., 2015). The salt tolerance mechanism involves multigenic traits acting together.

2.2.1 Ion homeostasis in salt tolerant plants

The salt tolerant plants, also called halophytes, have developed mechanisms that enable them thriving under saline conditions and act by excluding salt from their cells or by tolerating its presence within the cells. However, salt-sensitive glycophytes are not adaptable to the saline conditions due to the poor efficiency with water and solute accumulation. Geissler et al. (2000) suggested that salinity increases the Na^+ influx and inhibits K^+ uptake, which disturbs the homeostasis of K^+. The excessive salt concentration can't be tolerated by glycophytes, whereas halophytes have devised tolerance mechanisms like compartmentalization of ions in vacuoles executed by Na^+ /H^+ antiporters and salt extrusion through Na^+-antiporter (SOS1) present in plasma membrane. The presence of Na^+/H^+ antiporter in the plasma membrane of plants is critical for their growth under high salinity as it removes toxic Na^+ from the cytoplasm. Salt stress increases the activities of plasma membrane H^+-ATPase and Na^+/H^+ antiporter (Zhang et al., 2006). SOS1 gene from Arabidopsis was the first plasma membrane Na^+/H^+ antiporter cloned in higher plants (Shi et al., 2000). The overexpression of SOS1 in transgenic plants significantly improved salt tolerance in Arabidopsis, while mutant plants lacking SOS1 protein were extremely sensitive to salt stress (Zhu et al., 1998). Other mechanisms to curb the damage due to salt stress such as ROS detoxification and osmotic adjustments through osmolytes are discussed in details in sections below.

2.2.2 ROS detoxification

The salt stress is also one of the main reasons for the accumulation of ROS such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ion (O_2^-), and hydroxyl radical ($^{\bullet}OH$) (Hasegawa et al., 2000). The cellular damage is maximized by the hydroxyl radical formed by the intermediates of superoxide and hydrogen peroxide generation reactions (Foyer et al., 1997). In the absence of a scavenger for hydroxyl radicals, the oxidative stress is tackled only through keeping a check on its generation. The capability of plants to scavenge the ROS is a vital aspect of tolerance as ROS causes irrevocable damage to plants by disrupting cellular homeostasis. The concentration of ROS is controlled by two main routes; enzymatic and non-enzymatic mechanisms (Birben et al., 2012).

The enzymatic antioxidants such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), dehydroascorbate reductase (DHAR), ascorbate peroxidase (APX), and glutathione reductase (GR) induce a high level of stress tolerance in plants. Catalase (CAT) and ascorbate peroxidase (APX) are two major enzymes involved in H_2O_2 detoxification. CAT catalyzes the dismutation of H_2O_2 to H_2O and O_2 while APX catalyzes the formation of monodehydroascorbate by consumption of H_2O_2, thereby, protecting the plant from its deleterious effects (Jung, et al., 2000). It has been reported that the expression of ROS-scavenging enzymes increased under saline conditions in salt-tolerant cotton (Meloni et al., 2003), shoot cultures of rice (Fadzilla et al., 1997), cucumber (Lechno et al., 1997), but decreased in wheat roots (Meneguzzo et al., 1999).

The non-enzymatic antioxidant systems include ascorbate and glutathione (GSH), tocopherol, flavonoids, alkaloids, and carotenoids (Bose et al., 2014). Mutants with decreased ascorbic acid levels (Conklin et al., 1996) and GSH content (Creissen et al., 1999) are more prone to abiotic stress. ROS scavenging facilitates the maintenance of a high ratio of reduced to oxidized ascorbate and glutathione in cells. The oxidation of ascorbate forms dehydroascorbate, monodehydroascorbate, and glutathione oxidized gluatothione (GSSG). The reduction of glutathione and ascorbate drives energy from NADPH and is executed by glutathione reductase (GR), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR) using NADPH as reducing power (Tsugane et al., 1999). Ascorbate and glutathione levels are replenished by reduction of their oxidized forms GSSG, monodehydroascorbate (MDA), and dehydroascorbate (DHA). Under abiotic stresses such as

heat shock, cold temperature, drought, salinity, and biotic stress due to pathogen attack, the activity of GSH biosynthetic enzymes increases (Vanacker et al. 2000), which ultimately increase GSH levels (Noctor et al., 2002). However, the balance of antioxidants must be precisely regulated because, in chloroplasts, enhanced glutathione generation alters the overall redox state causing oxidative stress (Creissen et al., 1999).

2.2.3 Osmotic adjustment

One of the strategies employed by plants to counteract water deficit is through osmotic adjustments. For osmotic adjustment using organic osmolytes, plants need to synthesize and accumulate low molecular weight organic osmolytes (compatible solutes), which are neutral at physiological pH. These osmolytes have been categorized into four major classes: 1) sugars (trehalose), 2) polyols (glycerol, inositols, mannitol, sorbitol), 3) amino acids (proline, glycine betaine), and 4) quaternary ammonium compounds (β-alanine betaine, choline-O-sulfate, proline betaine, and hydroxyproline betaine (Rontein et al., 2002).

The osmolytes do not interfere with normal biochemical reactions and execute their highly specific protective mechanisms by maintaining the membrane structures and integrity of the enzymes under water deficit. For example, mannitol enhances tolerance to water-deficit stress primarily through osmotic adjustment and scavenging of OH^-, thus protecting ferredoxin and glutathione from the effects of hydroxyl radicals (Shen et al., 1997).

The response of proline to water deficits has been demonstrated in diverse plant species (Kavi Kishor et al., 1995; Nanjo et al., 1999; Hong et al., 2000). Transgenic plants with pyrroline-5-carboxylate reductase (P5CS) gene, which interconverts glutamate, ornithine, and proline in the antisense direction leading to suppression of proline synthesis, demonstrated sensitivity to water deficit (de Ronde et al., 2000). The osmotic adjustments can also be accomplished in salt tolerant plants by accumulating inorganic ions such as Na^+ and Cl^- as was observed in the halophyte quinoa (Hariadi et al., 2011). Halophytes have been observed to accumulate high Na^+ levels in vaculoles away from metabolically active organelles with vacuoles helping in osmoregulation (Wyn Jones & Gorham, 2002).

2.2.4 Control of xylem Na^+ loading and Na^+ retrieval from the shoot

Of the many salinity tolerance mechanisms, regulation of xylem loading and ionic exchange at the boundary of xylem and parenchyma is one of the most significant (Berthomieu et al. 2003; Munns & Tester 2008). The buildup of Na^+ in the shoot at toxic level can be tackled by restriction of Na^+ loading in xylem or increasing the retrieval of Na^+ from xylem so that Na^+ is kept away from the salt-sensitive shoot. In *sos1* mutant of Arabidopsis, which is incapable of controlling radial transport of Na^+, a five-fold increase in Na^+ concentration in xylem and shoot has been recorded (Nublat et al., 2001). The HKT1 controls the retrieval of Na^+ from the xylem. When it is overexpressed in *Arabidopsis thaliana* root stele, it reduced the accumulation of Na^+ by 37 to 64 percent in the shoot (Moller et al., 2009). The HKT transporters are suggested to be encoded by two dominant loci - Nax1 and Nax2 in wheat, and their expression linked to low Na^+ in leaf blade under salt stress (Davenport et al. 2007). On the other hand, the transgenic plants of barley overexpressing HvHKT2-1 transporters had high levels of Na^+ in xylem and better tolerance to salt stress in comparison to wild types (Mian et al., 2011). The reported content of Na^+ in the xylem of halophytes is around 50 mM, which is toxic and damaging to glycophytes even when the same amount is present in the soil (Shabala and Mackay 2011). This observation questions the notion of fewer Na^+ in the xylem imparting better salt tolerance in all plants. Na^+ has been suggested to contribute to the osmotic adjustment (Shabala et al., 2010). However, the increased concentration of Na^+ in xylem for longer duration is detrimental. Similarly, Na^+ sequestration in vacuole is limited. Therefore, the effect of Na^+ accumulation in xylem vessels of the plant is time dependent. The entry of the salt into the xylem vessels is through both symplastic and apoplastic pathways. Casparian strip blocks the apoplastic pathway and salt is forced to enter the symplasm. The salt loads up in xylem stream after permeation through cortical cells. Parenchyma cells of xylem control the loading and reabsorption of Na^+ from transpiration stream as being seen in *Phaseolus coccineus* (Kramer et al., 1977), maize (Johanson and Cheeseman,1983) *Glycine max* (Lauchli and Wieneke,1979), and bread wheat (Byrt et al., 2014). It has been observed that xylem parenchyma in proximity to bordered pitted xylem has protuberances in wall and act as transfer cells. The xylem parenchyma cells absorb Na^+ in exchange of K^+ from transpiration stream under salinity that has been confirmed by various analytical techniques (Lauchli and Wieneke,1979). However, controversy exists regarding the amount of Na^+ considered being safe to be taken by the roots and loaded into the transpiration stream and transported to shoots.

2.3 Nitric oxide signalling in plants

The ubiquitous signaling properties of nitric oxide (NO) in the animal kingdom have motivated the research in the area of its generation and action mechanisms since 1980, which got intense after Delledonne et al. & Durner et al. (1998) discovered the importance of NO in signal transduction in plants. Several characteristic features of NO such as lipophilic, with no charge, aids it in diffusion through the membranes and being free radical. It readily reacts with metalloenzymes and ROS regulating signalling pathways (Neill et al., 2003; Delledonne, 2005; Simpson, 2005). NO belongs to the family of reactive nitrogen species (RNS) comprising S-nitrosoglutathione (GSNO), peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3). These mentioned RNS execute post-translational modifications through nitration (Corpas et al., 2013) and S-nitrosylation of proteins (Astier & Lindermayr, 2012).

S-nitrosylation has emerged as an important NO-dependent post-translational modification in which NO group forms nitrosothiol by reacting with cysteine residues, hence regulating a wide array of cellular proteins. The covalent linkage of SH group of cysteine in target protein to NO forms S-nitrosothiol (SNO) (Lindermayr et al., 2006).

With the aid of biotin switch techniques, S-nitrosylated proteins have been identified. The nitrosylation of cysteine residues is a defensive mechanism adopted by plants against the protein carbonylation due to reactive oxygen species (ROS). The application of NO donor, like sodium nitroprusside (SNP), suppressed protein carbonylation in citrus plants subjected to the salt stress (Tanou et al., 2009). Enhancement in the germination was evident in *Antiaris toxicaria,* in the presence of gaseous NO (Bai et al., 2011). This is due to the nitrosylation of ascorbate-glutathione pathway, interrupting protein carbonylation, which causes loss of protein function. Programmed cell death, photosynthesis, regulation of phytohormones, and stress response elicited by the array of S-nitrosylated proteins are observed in plants (Astier and Lindermayr, 2012).

Tyrosine nitration is the modulation of proteins by targeting centers comprising transition metals resulting in the formation of peroxynitrite. It is an addition of the nitro group to one of the two equivalent ortho-carbons of the aromatic ring of tyrosine residues. Tyrosine nitration is a selective process in which one or two residues in the peptide may preferentially be nitrated relying on protein structure and location of the protein. N-Tyr incorporation into

proteins affects the enzymatic activity, brings changes in proteolytic degradation, impacts protein phosphorylation, and immunogenicity (Radi, 2012).

Along with S-nitrosylation, which is commonly recognized as a post-translational modification, tyrosine nitration is also called nitrotyrosylation. Tyrosine nitrotyrosylation has a role as regulatory protein modifier in animals and plants (Wimalasekera et al., 2011). Tyrosine nitration occurred in *Olea europaea* leaf proteins extract and in *Nicotiana tabacum* BY2 cell line suspension culture on exposure to high salinity (Valderrama et al., 2005).

2.3.1 Nitric oxide generation in plants

Different pathways synthesize the NO in plants; some of which involve various cellular enzymes and some occur without their aid. Identification of NO source and localization is necessary to have deep insight into its signaling pathways. Using fluorophore diaminofluorescein diacetate, it has been concluded that the physiological conditions of a plant influence the site NO generation (Planchet and Kaiser, 2006). Water deficit along with high-temperature conditions induce NO generation in the guard cells as shown in tobacco leaves (Lamotte et al., 2004). Hypocotyls of sunflower, when subjected to mechanical stress, led to NO synthesis in epidermal cells (Chaki et al., 2009).

One of the enzymes involved in NO production is nitrate reductase (NR). It is the first established scenario of enzymatic pathways for NO generation. The NR catalyzes the reduction of nitrate to nitrite by NAD(P)H and further to NO (Yamasaki et al., 2001). Through the genetic approaches, NR is believed to be the most significant enzyme involved in NO generation that is further required in plant signaling and immunity (Desikan et al. 2002).

Another enzyme capable of producing NO is nitric oxide synthase (NOS), which has been demonstrated to be present in plant peroxisomes (Chaki et al., 2009). Functional NOS has been identified in green alga *Ostreococcus trait of*, whose structural features include oxygenase and reductase domain linked by calmodulin (CAM) binding motif. Xanthine oxidoreductase (XOR) is an enzyme also shown to produce NO (Harrison, 2002), occurring in interconvertible forms of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) in cytosol.

The engagement of polyamines in the NO generation is observed as an addition of polyamines such as spermidine and spermine to Arabidopsis. Seedlings enhanced the NO generation in the elongation zone of the root tip and in primary leaves (Tun et al., 2006).

2.3.2 Effect of nitric oxide in plant hormone-mediated signalling

The NO regulates several processes in plants such as seed germination, flowering plants along with defense response to abiotic and biotic stresses (Lamattina et al., 2001; Neill et al., 2002; Delledonne, 2005). NO relays its effect in plants through modulation of secondary messengers, protein kinases, and plant hormones (auxin, abscisic acid, and cytokinin). For example, NO and cGMP are downstream effectors of auxin in root organogenesis (Pagnussat et al., 2002, 2003).

The gibberellins synthesis and signal transduction is also regulated by NO (Bethke et al., 2007). Seed germination was found to be regulated by NO and gibberellins (Beligni et al., 2002). Similarly, NO and gibberellins were found to inhibit hypocotyls elongation during seedling de-etiolation. Indole acetic acid (IAA) application to cucumber explants caused a temporary increase in NO in the basal region of the hypocotyl (Pagnussat et al., 2002). It is possible that NO produced in high concentrations in a confined area stimulates guanylyl cyclase (GC)-catalyzed synthesis of cGMP, thereby promoting adventitious root formation. This is supported by the fact that application of GC inhibitor causes a decline in the development of adventitious roots, in spite of treatment with IAA or NO in cucumber explants (Pagnussat et al., 2002).

2.3.3 Implications of nitric oxide signalling in plants under saline conditions

NO induces several physiological modifications in the plant to counteract salinity. The most important modifications are the induction of stomatal closure with the aid of abscisic acid (ABA), the regulation of photosynthetic activity, and ion homeostasis. Exogenous application of NO induced the expression of plasma membrane H^+-ATPase in plants under salt stress, which is crucial for Na^+ exclusion through SOS1 (Silva and Gerós, 2009).

The NR-dependant NO production has shown to improve salt tolerance in red kidney bean (Liu et al., 2007). The NO-induced ROS scavenging enzymes such as SOD, POD, and CAT, which suppressed the rate of ROS damage (Guo et al., 2009). Moreover, NO also was shown to induce the expression of stress-related genes encoding sucrose-phosphate synthase and Δ′-pyrroline-5-carboxylate synthase (Uchida et al. 2002). Xu et al. (2011) observed that several salt-induced genes (AcGH3.3, AcTIP1, and AcTIP2) in dimorphic seedlings of *Atriplex centralasiatica* were regulated via an NO-dependent process highlighting the key role of NO in the regulation of genes related to salt tolerance. The transcripts of these genes are involved in activation of antioxidant enzymes and regulation of water and ion uptake.

2.3.4 Induction of stomatal closure

Various studies have observed an NO synthesis and accumulation in guard cells under water stress (Magalhaes et al., 2000). Indeed, experiments have confirmed a strong correlation between NO synthesis in guard cells and increased ABA production during water stress (induced by polyethylene glycol) (Zhang et al., 2007). The application of exogenous NO in *Vicia faba*, *Tradescantia* spp, and *Salpichroa organifolia* induced stomatal closure and consequently reduced the transpiration rate (Garcia-Mata and Lamattina, 2003). ABA-induced closure of stomata due to NO generation was impaired in the *Atnos1* and *nia1nia2* mutants deficient in (NIA) genes, which code for nitrate reductase (Desikan et al., 2002). The NO donor – sodium nitroprusside-induced stomatal closure was shown to be concentration and time-dependent. Moreover, its impact can be averted by the action of NO scavengers such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Bright et al 2006). The inhibition of NO generation in guard cells with the treatment of either L-NAME (N-nitro-L-Arg-methyl ester; NOS inhibitor) or tungstate in Arabidopsis leaves resulted in the stomatal opening (Neill et al. 2002). There is a possibility of variation in the role of NO relying on its concentration and the physiological condition of guard cells. It was observed that NO donor (i.e. SNP) was responsible for stomatal opening when applied at high concentrations (0.5– 1 mM) (Sakihama et al., 2003) contrary to low levels (0.1–50 µM) of SNP, which were ineffective to initiate stomatal opening (Bright, 2006).

2.3.5 Regulation of programmed cell death

PCD has a key role to play in conferring adaptation to the change in environments such as cold stress, waterlogging, salinity, and hypoxia (Katsuhara and Kawasaki, 1996). Salt stress has been seen to induce PCD in primary roots of both wild-type and salt overly sensitive (*sos1*) mutant seedlings of Arabidopsis. However, due to the formation of secondary roots from the transition zone, the wild type survived, but *sos1* mutants died (Huh et al., 2002). PCD is marked by the appearance of characteristic biochemical changes such as the nucleus and cytoplasm condensation, DNA laddering, and formation of apoptotic bodies. Other features of PCD are high caspase-like proteolytic activity and release of cytochrome *c* from mitochondria, although genes encoding caspase genes are not found in plants. Caspase analogous protease activity has been observed in tobacco cell suspension culture subjected to pathogen-induced PCD (Chichkova et al., 2004). An increase in caspase-like activity was observed in NO-treated plant cells (Clarke et al., 2000).

The influx of Na^+ through nonselective cationic channels (NSCC) under saline conditions showed to cause a significant membrane depolarization leading to massive K^+ efflux from the cells through KOR channels (Shabala et al., 2006). Salinity also stimulated elevation in cytosolic Ca^{2+}, which resulted in the sudden increase of ROS levels, thus leading to enhanced K^+ efflux through NSCC (Tracy et al., 2008). The net decline in cytosolic K^+ concentration was reported to activate caspase-like proteases executing PCD (Demidchik et al., 2003).

Salinity-induced PCD was found to be linked to disturbed ion homeostasis of Ca^{2+}, K^+, and H^+, and increased production of hydrogen peroxide as shown in several plant species (Bose et al., 2014; Jayakannan et al., 2015). *Caenorhabditis elegans* anti-apoptotic gene- CED-9 expressed in tobacco showed to improve the maintenance of K^+ homeostasis and protection from salt-induced cell death (Shabala et al., 2007). Another study showed the role of H_2O_2 and NO in manipulating pathogen-induced PCD (Delledonne, 2005). Plant NO synthase contains a calmodulin (CAM) binding motif. Therefore, both Ca^{2+} and CAM are required for its optimal activity (Guo Crawford, 2005). In turn, NO participates in amplifying Ca^{2+} signals (Lamotte et al., 2004). NO levels are important determinants of PCD. In Arabidopsis cell cultures, the NO-stimulated cell death was similar to the one induced by H_2O_2 (Van Breusegem and Dat, 2006). However, NO stimulated cell death requires longer exposure initiating RNA processing and

protein synthesis. This suggests that NO is not directly toxic to the cells, but activates some other cellular processes that lead to PCD (Río and Puppo, 2009).

2.3.6 Nitric oxide stimulates alternative respiratory pathway during salinity stress

Plant mitochondria have an electron transport pathway supplementary to the cytochrome pathway (CP) called the alternative respiratory pathway (AP), which plays a key role in fruit ripening, thermogenesis, and environmental stress acclimation (Ederli et al., 2006).

The induction of AP by ethylene and H_2O_2 has been reported in Arabidopsis (Simons et al., 1999). H_2O_2 is considered the second messenger, which regulates alternative oxidase (AOX), the terminal oxidase in an alternate pathway through oxidization of transcription factors or modulation of phosphorylation process (Neill et al., 2002). Although ethylene and H_2O_2 have been observed to be engaged in AP induction, the ambiguity remains in the interaction between them in the commencement of the AP during environmental stresses. Salt stress has also shown to stimulate the action of AP through ethylene emission (Smith et al., 2009).

The salt stress has been observed to cause NO accumulation in the callus of Arabidopsis, by stimulating nitric oxide synthase (NOS), triggering 1-aminocyclopropane-1-carboxylic acid synthase (ACS) activity, which in turn leads to the ethylene emission (Wang et al., 2009). The growing evidence suggests that stimulation of plasma membrane NADPH-oxidase activity by NO generated under stress due to excess of salt, account for the production of H_2O_2 (Zhang et al., 2007). The accumulated H_2O_2 activates ACS, which further leads to an increase in ethylene emission, thus modulating AOX1a expression. Therefore, AP activity gets amplified, hence causing a reduction in the H_2O_2 generation and averting ROS damage in plant cells (Huang et al., 2002). By comprehending these observations, the role of NO in H_2O_2 and ethylene-dependent AP induction can be assumed under saline conditions.

2.3.7 Enhancement in chlorophyll and photosynthetic activity

Photosynthesis is one of the elementary processes impaired by salinity (Munns et al., 2006). The effects can be primarily due to reduced CO_2 uptake caused by diffusion limitations through the stomata, or secondarily due to the occurrence of oxidative stress affecting leaf

photosynthetic machinery (Flexas et al., 2007). It was shown in experiments with sorghum and rice that the quantum yields of photosystem II did not change substantially, but the electron transport rate under saline conditions decreased, and photochemical quenching increased significantly. The increase in photochemical quenching reflects a reduced demand for products of electron transport, resulting in an increased heat dissipation, thus affecting the photosynthetic apparatus adversely (Netondo et al., 2004; Dionisio-Sese and Tobita, 2000).

It has been reported that high concentration of NO in chloroplasts results in the generation of reactive nitrogen species (NOO^{-}) leading to diminished photosynthesis (Jasid et al., 2006). The chloroplast electron transport is inhibited by NO in a reversible manner without altering the quantum efficiency. The resultant pH gradient across the thylakoid membrane decreases the rate of ATP synthesis (Takahashi and Yamasaki, 2002). Photorespiration along with photosynthesis has also been found to be affected by NO in different plants due to the effect of NO on stomatal behavior (Yamasaki and Sakihama, 2000). On the other hand, pre-treatment with SNP (NO donor) improved the rate of photosynthesis and chlorophyll content in cucumber seedlings (Fan et al., 2007). The SNP has been seen to reduce the Rubisco activity level and the ß-subunits of the Rubisco subunit-binding protein in mung bean (Lum et al., 2005). From above observations, it appears that maintenance of optimal NO concentration inside the chloroplasts is critical for optimal photosynthesis.

2.3.8 Role of nitric oxide in ion homeostasis during salt stress

Maintenance of optimal intracellular K^+/Na^+ ratio is crucial for plant survival under salinity stress. Plants extrude excessive Na^+ into apoplast or compartmentalize into vacuoles (Apse et al., 1999) while retaining the physiological K^+ level in the cytoplasm. The cytoplasmic Na^+ extrusion activity is executed by plasma membrane bound and vacuolar Na^+/H^+ antiporters (Blumwald et al., 2000). The energy for Na^+ extrusion is derived from the plasma membrane H^+-ATPase (PM H^+-ATPase) and vacuolar H^+-ATPase (V-H^+-ATPase) (Niu et al., 1996).

Salt overly sensitive-1 (SOS1) gene encodes PM Na^+/H^+ antiporter and confers salt tolerance in plants (Zhu, 2001). The vacuolar Na^+/H^+ antiporters belong to the NHX1 family. SOS1/NHX overexpression decreases Na^+ accumulation in the cytoplasm and enhances salt tolerance in transgenic plants of Arabidopsis, tomato, and mustard (Shi et al., 2003).

NO regulates the activity of V-ATPase by influencing the Ca^{2+} influx, which further activates SOS2 to form a complex with SOS3. During salt stress conditions, the SOS2-SOS3 complex phosphorylates and activates the transport activity of the SOS1 antiporter (Quintero et al., 2011). Moreover, SOS2 directly interacts with V-ATPase regulatory subunits B1 and B2 hence switching it on to create proton gradient (Chen et al., 2010). NO has been observed to significantly increase the salt tolerance of maize seedlings by stimulating vacuolar H^+-ATPase and ultimately Na^+/H^+ antiporter in the tonoplast (Zhang et al., 2006). In the reed callus, the same mechanism of Na^+ efflux out of cytoplasm is reported (Zhao et al., 2004). Guo et al. (2008) treated *Kosteletzkya*with NaCl (100 and 200 mM) and later with NO donor sodium nitroprusside (SNP). The application of SNP significantly decreased the intracellular level of Na^+ in shoots and roots of *K. virginica* seedlings under salt stress.

Transient increase of nitric oxide level in leaves of maize in response to application of NaCl suggests that NO and NaCl treatments activate the V- H^+-ATPase and H^+-PPase, thus increasing the H^+-translocation, which results in $Na^{+/}H^+$ exchange (Zhang et al., 2006). Phospholipase D associated with the plasma membrane showed to trait of by NO that is generated under salt stress. This enzyme catalyzes the hydrolysis of phospholipids present in the membrane, hence generating polyamines. The signaling cascades of polyamines induced tonoplast H^+-ATPase activity is well documented (Testerink and Munnik, 2005). SNP treatment has been observed to attenuate the side effect of Na^+ toxicity in *Atnoa1* (nitric oxide synthase gene) mutant plants (Zhao et al. 2007).

Chen et al. (2010) experimentally clarified that NO plays a pivotal role in achieving lower Na^+/ K^+ ratio in the cytosol by introducing various concentrations of SNP in mangrove plant *Avicennia marina*. The optimal concentration of SNP enhanced Na^+ extrusion into salt glands by activating SOS1, along with increased vacuolar Na^+ sequestration by stimulating VHA-c1 and NHX1 genes.

The stimulation of guanylate cyclase enzyme by NO aids in the biosynthesis of secondary messenger cGMP. The cGMP along with NO-mediated S-nitrosylation affects the gating of Ca^{2+}-dependent and Ca^{2+}-independent K^+ channels (Bolotina et al., 1994), as well as Na^+ channels (Renganathan et al., 2002). Salt-induced K^+ efflux through outward rectifying K^+ channels can be restricted by inhibiting depolarization of membrane by salt stress through increased H^+ efflux (Sun et al., 2007). Enhanced H^+-ATPase activity is essential for

maintenance and repolarization of the membrane potential (Palmgren and Nissen, 2010). It was found that salinity tolerance among barley genotypes is determined by H^+-ATPase activity (Chen et al., 2007). As NO up-regulates plasma membrane H^+-ATPase activity during salinity stress (Shi et al., 2007), it is possible that NO would play a huge role in K^+ retention during salt stress. In addition, NO has also been found to have a reversible negative influence on the K^+ outward channels as these channels open in the presence of reducing agents (2-3 di mercapto propanol) (Sokolovski and Blatt, 2004) and salt (Jayakannan et al., 2013) by inducing nitrosylation of Cys residues present in these ion channels.

The K^+ inward-rectifying channel, AKT1, is involved in K^+ uptake from soil into the root epidermal cells (Corratgé-Faillie et al., 2010). The expression of the OsAKT1 in rice (a glycophyte) was down-regulated under salinity stress (Fuchs et al., 2005). However, in *Puccinellia tenuiflora* (a halophyte) PutAKT1 transcriptional level remained unaffected by the high concentration of NaCl (Ardie et al., 2010). Transgenic *Arabidopsis* expressing PutAKT1 had significantly higher resistance to high salinity (Ardie et al., 2010). In another study, the transcriptional expression of AKT1 was up-regulated in the roots of NaCl-treated mangrove *Kandelia obovata* (Berthomieu et al., 2003). The application of NO donor SNP was found to up-regulate the expression of AKT1 in the roots of NaCl-treated *K. obovata*, suggesting the significance of NO in increasing K^+ uptake in salt-treated roots.

2.3.9 Modulation of transcription factors by NO

The available transcriptomic and proteomic data reveal that NO can influence the expression of various genes and proteins involved in physiological processes (Polverari et al., 2003). However, identification and characterization of specific target genes and proteins influenced by NO in salt-stressed plants are still at a rudimentary stage. In citrus plants, the proteins controlled by NO during salt stress have been elucidated (Tanou et al., 2009). Root pre-treatment with SNP reversed the induction of NaCl-responsive proteins involved in the Calvin-Benson cycle, glycolysis, and defense mechanism. These proteins are potentially necessary for inducing salt tolerance as they carry out NO-mediated priming effect by acclimatizing citrus plants to salinity. In *Arabidopsis thaliana*, NOinduced changes in the expression profiles of 2500 transcripts. Among these transcripts, 120 of them were mainly involved in signal transduction executing defense, ROS generation/removal, photosynthetic processes, and PCD (Polverari et al., 2003). The microarray results also concluded that NO-

regulated genes are affected by other abiotic/biotic stresses (Parani et al., 2004). The whole-genome microarray of *A. thaliana*, involving approximately 24,000 genes, showed that 342 genes were up-regulated and 80 genes were down- regulated by NO (Parani et al., 2004). The modulation of various transcription factors was shown to be stimulated by NO (Polverari et al., 2003). These include ethylene-responsive element binding proteins (EREBPs) and dehydration-responsive element binding proteins (DREB1 and DREB2) along with transcripts coding for oxidative stress-related proteins (GSTs, ABC transporters), iron homeostasis proteins (ferritin genes), and signal transduction factors (e.g. MAP kinase modules).

2.3.10 Crosstalk between polyamines and nitric oxide

Polyamines (putrescine, spermidine, and spermine) are polycationic aliphatic compounds ubiquitous to plants and animals that are involved in fundamental metabolic processes such as DNA replication, protein synthesis, ROS scavenging, and maintenance of ion homeostasis in cells (Takahashi and Kakehi, 2010; Pottosin et al., 2014). Under biotic and abiotic stresses, levels of polyamines rise (Gupta et al., 2013). This increase in the endogenous concentration of polyamines has a beneficiary effect on plant performance under stress. This was further supported by employing gain or loss of function mutants for biosynthesis of polyamines (Alcázar et al., 2010). Due to their polycationic nature, they have a tendency to bind to negatively charged biomolecules and membranes and act as chaperons (Galston and Kaur Sawhney, 1990; Kusano et al., 2008). They also contribute to ROS detoxification by acting as free radical scavengers and activating antioxidant enzymes (Ha et al., 1998; Das and Misra, 2004; Zepeda et al., 2011). The non-selective cation channels, as well as K^+ outward rectifying channels at the plasma membrane, are inhibited by polyamines, thus restricting Na^+ influx and K^+ efflux (Shabala et al., 2007; Zhao et al., 2007). The aforementioned effects limit's plasma membrane depolarization, optimise K^+/Na^+ ratio, and hence reduce the negative effects of salt stress (Zepeda-Jazo et al., 2008). Polyamines also cross talk with NO (Tun et al., 2006; Arasimowicz-Jelonek et al., 2011) and thus influence aforementioned responses.

NO and polyamines have been suggested to interact with several cellular processes (Neill et al., 2003; Li et al., 2014). For example, both NO and polyamines increase the levels of antioxidants enzymes in plants under stress (Alcázar et al., 2010). The excessive NO produced during severe stress showed to be scavenged by the conjugate compounds of polyamines, and cytotoxicity posed by NO was tackled. Ascorbate-glutathione

recycling, which elevates abiotic stress tolerance was shown to be enhanced by the exogenous application of NO and polyamines (Lin et al., 2012).

The synthesis of NO is also increased following the application of polyamines and hydroxylamine (Silveira et al., 2006; Tun et al., 2006; Wimalasekera et al., 2011). Additionally, the application of NO donor sodium nitroprusside (SNP) has been observed to regulate polyamine metabolism in *Medicago truncatula* leaves (Filippou et al., 2013). These findings suggest that polyamines induce their physiological effect by altering the endogenous concentration of NO (Fröhlich and Durner, 2011).

2.4 Cytokinin (CYT) in plant development and adaptation to salt tolerance

CYT responds to the abiotic stress by evoking few signaling pathways (Ha et al., 2012; Hwang et al., 2012; Zwack and Rashotte, 2015). Many studies reported a decrease in endogenous CYT concentration under abiotic stress led to stress tolerant phenotype (Kudoyarova et al., 2007; Merewitz et al., 2011; Nishiyama et al., 2011). In contrast, a rise in the endogenous levels of CYT and overexpression of biosynthesis genes - adenosine phosphate-isopentenyl transferases (IPTs) has been reported in plants under severe stress (Dobra et al., 2010). Kinetin, one of the types of CYT, is known to enhance the growth of crop plants significantly under salinity, soil waterlogging and soil acidity (Gadallah 1994; Salama and Awadalla 1987). Similarly, enhanced levels of zeatin have been reported in few Mediterranean shrubs under salinity stress (Lopez-Carbonel et al. 1996). The salt resistant barley with stunted growth under salt stress showed reduced levels of zeatin (Kuiper et al., 1990). Exogenous application of gibberellins and CYT aided plants to grow in NaCl rich soil. The mechanisms involved in conferring salt tolerance by CYT have been discussed in following sections.

2.4.1 Cytokinin metabolism

Cytokinin is a derivative of adenine with either aromatic chains or isoprenoid attached. Isoprenoids containing CYT such as isopentenyl adenine (iP)-, dihydro zeatin, trans, and cis-zeatin are prevalent in plants in comparison with an aromatic side chain containing CYT such as N6- (meta-hydroxy benzyl) adenine (Strnad et al., 1997; Sakakibara, 2006). The synthesis CYT, being an isoprenoid, is limited by the activity of isopentenyl transferases (iPTs). The

addition of isopentyl by IPTs to the adenine moiety requires ATP and tRNA (Kakimoto, 2003; Brugiere, et al., 2008).

The riboside phosphates of isopentenyl adenine and trans-Zeatin (tZ) are converted into bioactive CYT in two steps via removal of the phosphate group and then the ribosyl moiety. The removal of the ribosyl and phosphate group is executed in a single step by the CYT riboside 50-monophosphate phosphoribo hydrolase (LOG) (Kurakawa, et al., 2007; Kuroha, et al., 2009). Overexpression of AtLOGs had enhanced the levels of isopentyl and their glucosides, thus resulting in apical dominance and delay in senescence. Cytochrome P450 monooxygenases catalyze the hydroxylation of methyl groups present in the side chain of isopentyl to form tZ. The generation of biologically active iP-type CYT and tZ by IPTs is localized to the chloroplast. iP CYT shows basipetal movement via phloem to (Matsumoto-Kitano et al., 2008).

The degradation of CYT by CYT dehydrogenase, which removes the isoprenyl chain from CYT, has a significant regulatory role to play in CYT degradation. These CYT-degrading enzymes localized in the cytosol, vacuoles, and apoplast has higher substrate affinity for iP and tZ as compared to ribosides (Werner et al., 2006). The CYT level decreases with overexpression of CYT dehydrogenase (CYTX), which suppresses shoot growth and enhances root development. More prominent phenotypic changes are evident with the overexpression of CYTX1, CYTX3, and CYTX5 in comparison to other CYTX genes (Werner et al., 2003). The critical role of the CYT in reproductive development in plants is evident in CYTX3 CYTX5 double mutant showing supernumerary ovules, larger inflorescence, and increased seed yield (Bartrina et al., 2011). The deactivation of the CYT is also achieved through glycosylation, mainly by glucose. The attachment of the glucose to the adenine ring of CYT at N7 or N9 position results in irreversible deactivation, while attachment at N3 or OH group is reversible, hence making CYT suitable for storage (Frebort, et al., 2011).

2.4.2 Cell cycle control, chloroplast development and photomorphogenesis

CYT plays a significant role in the regulation of cell cycle. It has been suggested by several studies that CYT are responsible for a transition from G2 to M phase of mitotic replication in plants (Francis, 2011; Lipavská et al., 2011). The expression of cdc2 (Cell division control 2) gene, which codes CDK1; a cell cycle regulator protein, is observed to be

induced by CYT in Arabidopsis roots and other plant tissues (Hemerly et al., 1993). CYT treatment to the tobacco cell cultures resulted in enhanced activity of cyclin-dependent protein kinase (CDK) Tyr phosphatase at G2/M transition phase. CYT activates the CDK by dephosphorylation of Tyr by cyclin-dependent protein kinase (CDK) Tyr phosphatase, thus leading to progression through mitosis. The loss of function in *ahk2ahk3ahk4* triple receptor mutant had disrupted cell division, which resulted in the smaller shoot and root apical meristem. The delay in the G2/M transition under the limited supply of CYT in the plant has been demonstrated by fluorescence-activated cell sorting technique in which sorting of root tips showed that the number of 4N cells (tetraploid in G2/M) was higher than 2N cells (diploid in G1) (Higuchi et al., 2004).

The response of the seedlings to light, known as photomorphogenesis, has been mimicked by increasing the endogenous levels or exogenous application of CYT (Chory et al., 1994; Lochmanova et al., 2008). A two-component signaling of CYT activates photomorphogenesis. Red light photoreceptor (PhyB) interacts with Arabidopsis CYT response regulator- ARR4 stabilizing active form of PhyB known as Pfr. Hypersensitivity to the red light observed due to overexpressed ARR4 (Sweere et al., 2001). However, another study showed that the quadruple mutant *arr 3, 4, 5, 6* was more sensitive to red light compared to the wild-type seedlings suggesting type-A ARRs are negative regulators of phytochrome function (To et al., 2004). The loss-of-function mutant *arr4* was found to be less sensitive to the red light (Mira-Rodado et al. 2007). These results suggest multiple type-A ARRs are implicated in the regulation of phytochrome function.

Biogenesis of chloroplast is also one of the events of photomorphogenesis regulated by CYT. Overexpression of CRF2 (CYT response factor) up-regulates the PDV2 level (plastid division), which ultimately increases the chloroplast numbers (Okazaki et al., 2009). Chiang et al. (2012) indicated that GNC (gata nitrate-inducible carbon-metabolism) and CGA1 (CYT-responsive gata1) are the targets of the CYT. These factors mediate the development of chloroplasts from proplastids and increase division and growth of chloroplast. Exogenous application of CYT induced proplastids differentiation into chloroplast in cotyledons of pumpkin and watermelon (Khokhlova et al., 1978; Longo et al., 1979), chloroplast maturation in *Nicotiana tabacum* cell cultures, and *Nicotiana rustica* leaves (Zavaleta-Mancera et al., 1999). CYT also enhances photosynthesis by activating chloroplast enzymes (Kusnetsov et al.,

1998) and by inducing transcription of several photosynthetic genes encoded in the plastids (Zubo et al., 2008).

2.4.3 Cytokinin signaling pathway

The signaling of the CYT is relayed to the histidine kinases, which are membrane-bound sensors (Brandstatter et al., 1998). The signal of CYT is transduced via His–Asp phosphorelay similar to that two- component signalling in bacteria (TCS) (Schaller et al., 2011). The hybrid histidine kinases are receptors of CYT, which get auto-phosphorylated, and the phosphoryl group is transferred via histidine phosphor-transfer proteins to type-B response regulators. The response regulators are the last output element in two- component signalling and their activity changes as its phosphorylation state. There are two types of response regulators- positive (type B) and negative (type A) sensors, which regulate the primary response genes (To and Kieber, 2008). This quick cellular response doesn't require synthesis of new proteins. The type-A response regulators involved in negative feedback of CYT signaling have phosphorylation acceptor site, and their expression is stimulated by CYT treatment (Muller and Sheen, 2007). Rate limiting step in CYT signaling is played by B-type response regulator (Hwang and Sheen, 2001).

A study has demonstrated the location of Arabdopsis histidine kinase (CYT receptors) -AHK2, AHK3, and AHK4 in endoplasmic reticulum from where the signal is suggested to be initiated (Caesar et al., 2011). AHK4 behaves as a kinase in the presence of CYT, and in their absence, it acts as a phosphatase. This trait is missing in both AHK2 and AHK3 (Mahonen et al., 2006). A domain of CYT receptors known as cyclases/histidine kinases associated sensory extracellular (CHASE) has been suggested, by biochemical assays, to be essential for CYT binding. Two transmembrane domains flank this domain and mutations in these transmembrane domains or CHASE domain keep them active constitutively (Miwa et al., 2007).

2.4.4 Crosstalk between cytokinin and ABA

Abscisic acid and CYT can behave antagonistic and synergistic in plant's various processes depending on the prevailing environmental conditions. The interaction of CYT with other phytohormones, especially ABA and auxin, have been hypothesized to enhance the

tolerance to salinity in wheat (Iqbal et al., 2006b). ABA regulates the dehydration related processes under stressful and non-stressful conditions (Umezawa et al., 2010). The elevated levels of CYT downregulate the ABA-responsive genes whereas decreased CYT led to up-regulation of ABA hypersensitivity to the stress responsive genes (Nishiyama et al., 2011). The enhanced tolerance to salt stress has been attributed to the hypersensitivity to ABA in CYT mutants. The profound effect of the stress amelioration in these mutants was achieved due to the ABA-dependent AREB activation, which is ABA-responsive cis- element. (Tran et al., 2010). In response to osmotic stress, the majority of ABRE-dependent gene expressions were shown to be mediated by ABA. However, whether the enhanced levels of the ABA are playing a role in down-regulating CYT signaling remains to be resolved.

The transcriptome analysis of the stress signals suggested that low CYT content was due to down-regulation of IPT genes expression (Nishiyama et al., 2011). The exogenous application of ABA suggested the role of the ABA in down-regulation of the stress-induced suppression of CYT signaling. More experiments in ABA-deficient plants are needed to provide us with the exact role of ABA in CYT-induced stress response. An increase in the net CYT content downregulates both ABA and stress responsive genes (ABI5). The interplay between the CYT and ABA under stress is considered to be controlled by protein complex of the ABI5 and type-A response regulators 4, 5, 6.

2.4.5 Role of cytokinin in osmotic and salt stress

In a study by Lopez-Carbonel et al. (1996) on leaves of *Rosmarinus officinalis*, zeatin increased for three days of CYT treatment and started to decrease in response to salt stress. In transgenic *Sinorhizobium* overexpressing *ipt* gene, more CYT was generated, which improved tolerance to severe drought stress. The application of CYT at different concentrations along with varying treatment durations have been investigated to see its effect on the salinity tolerance (Munns and Termaat, 1986). Several studies have been conducted to understand the interactions between CYT and salicylic and jasmonic acid, ethylene, and ABA (Liu et al., 2013). Water deficit conditions were found to reduce the CYT levels in plants, but overexpression of isopentenyl transferase (IPT), an enzyme involved in CYT generation, alleviated this decrease, and hence enhanced stress tolerance (Havlová et al., 2008; Ghanem et al., 2008). The expression of IPT driven by SAG12 (senescence promoter) and SARK (maturation promoter) resulted in delayed photosynthetic decline due to age and reduced the

yield loss imposed by water deficit on crop plants (Xu et al., 2009; Rivero et al., 2007; Wingler et al., 1998).

The inhibition of leaf growth and premature senescence were linked with a decrease in concentrations of CYT in leaf, root, and xylem sap (Albacete et al., 2008; Ghanem et al., 2008). Similarly, increased CYT levels in xylem sap imposed by salinity influenced shoot responses that are conferred by CYT synthesized in roots (Albacete, 2008). The yield of transgenic tomato WT/35S IPT increased when exposed to salinity (Ghanem et al., 2011). The enhanced expression of IPT gene was shown to increase the transport rate of CYT from root to shoot, which resulted in increased vegetative and fruit growth and enhanced salt tolerance (Ghanem et al., 2011).

Exogenous application of kinetin stimulates K^+ influx in root hair cells by regulating K^+ permeable channels present in the root epidermis (Schumaker and Gizinski, 1993). Indeed, (Shabala et al., 2008) demonstrated that the uptake of K^+ into the cells is controlled indirectly by kinetin. K^+ channels are activated/deactivated by their hyperpolarization or depolarization, respectively, when kinetin is present in the root apoplast (Shabala 2003). At the same time, some studies found no effect of kinetin on the inward-rectifying K^+ channel (KAT1) present in guard cells (Mori et al., 2000).

The absorption of excess photons by light harvesting complexes of plants results in over reduction of electron transport chain leading to the generation of ROS. CYT amend abiotic stresses such as salinity, drought, and osmotic stress (Cortleven et al., 2014). Drought and salinity negatively impact on transpiration, solute transport and photosynthesis (Farooq et al., 2009). The CYT receptors-AHK2 and AHK3 histidine are expressed under osmotic stress indicating the pivotal role played by CYT under these conditions. The loss of function of both or one of the receptors results in enhanced drought tolerance as well as increased sensitivity to ABA (Tran et al., 2007). Osmotic stress has been observed to induce expression of AHK2 and AHK3 – CHKs in Arabidopsis indicating the involvement of CYT in stress response. Under salinity stress, AHK2 and AHK3 are stimulated, and it has been reported that *ahk2, ahk3* (single mutants) and *ahk2,3* double mutants were more tolerant to salt stress (Tran et al., 2007). Moreover, *arr1arr12,* a double mutant of response regulator protein of CYT was shown to enhance salt tolerance (Mason et al., 2010).

2.5 Research objectives

The extensive research has been conducted on the use of the nitric oxide and other phytohormones in conferring tolerance to abiotic/biotic stresses. However, to the best of my knowledge, these pivotal studies still have not resulted in salt tolerant cultivars in farmers' fields. This calls for a need to move from merely observational to mechanistic studies, to reveal the underlying physiological and molecular mechanisms behind the reported phenomena.

Accordingly, the main objectives of this study were as follows-:

a) To investigate the applicability and efficiency of using NO and CYT as ameliorative agents, to improve plant performance under saline conditions;
b) To understand the physiological basis of above ameliorative effects, both at the whole-plant and cellular level;
c) To link observed beneficial effects at whole-plant level with effects of NO and CYT on plasma membrane transport and maintenance of the optimal K^+/ Na^+ ratio in salinized plant tissues;
d) To investigate possible downstream targets for CYT and NO signalling; and
e) To link beneficial effects of CYT and NO treatment with plant oxidative status and sensitization/desensitization of specific membrane transporters.

Chapter 3: Effect of exogenous application of nitric oxide on growth and physiological characteristics of pea and barley plants grown under saline conditions

Abstract

The current study attempted to explain the ameliorative effect of nitric oxide (NO) donor sodium nitroprusside (SNP) on salinity leaf tissue tolerance in barley and pea. The combined effect of salt stress and foliar application of SNP (100 µM) on the plants has been evaluated through measurement of chlorophyll content and photochemical efficiency ($P< 0.005$) of PSII in leaves under both *in vitro* and *in vivo* conditions. Effect of SNP on plant agronomical characteristics and net CO_2 assimilation have also been studied in salinized plants under glasshouse conditions. The study has demonstrated a positive impact of NO in improving the chlorophyll content, and photochemical efficiency of barley and pea leaves in *in vitro* experiments ($P < 0.05$). However, outcomes of the *in vivo* experiment on whole plants were not similar to outcomes of the *in vitro* experiments. Uncertainties and challenges in ensuring efficient and uniform absorbance of SNP stray into the leaf tissue were most likely one of the main reasons that led to negating its efficacy when applied to intact plants. Therefore, application of NO in the field for equipping crop plants with salt stress resistance needs further review.

Introduction

Soil salinity is considered one of the most severe global environmental agricultural constraints. Over 17 million ha cultivated land is estimated to be affected by salt stress in Australia by 2050 (ANRA 2001). Salt stress influences growth of a plant by inflicting both ionic and osmotic stresses (Munns 2005). A higher concentration of salts in the soil makes roots inefficient to extract water. This leads to an increase in salt concentrations inside the plant, which can have toxic effects. Deposition of salts on the external surface of roots has an immediate effect on the growth of cell and associated metabolism; on the other hand, accumulation of lethal concentrations of salts inside plants before affecting plant function requires time (Munns and Tester 2008). Soil with higher salt concentrations in the upper stratum of soil in which seed is planted often leads to failure in germination (Fowler 1991).

The primary symptoms of the effect of salt stress on plant growth include; a decline in plant length, reduction in fresh and dry weight of plant parts, decrease in plant yield, and drop in the product quality (Ali-Dinar et al. 1999; Chartzoulakis and Klapaki 2000). Restrained photosynthetic ability of various plant species due to salt stress has been reported in many species (Makela et al. 1999; Hamayun et al. 2010; Kanwal et al. 2011). This decrease in the photosynthetic rate of plants under salt stress is related to the (a) reduction in permeability to CO_2 as a result of drying out of cell membranes; (b) decreased stomatal conductance as a result of limitation in supply of CO_2; (c) increased leaf senescence; (d) alteration in photochemical capacity and enzyme activity; and (e) repression in metabolic processes due to hindrance in CO_2 uptake.

Currently, there is a need for evolving strategies to counteract the growing concern of diminishing agricultural land and the yield loss due to salt stress. In many research studies, salt stress tolerance is induced in plants through genetic manipulations or seed retreatments and foliar sprays (Krishnamurthy 1991; Flowers 2004). Salt stress tolerance is introduced through genetic manipulation via transgenic and breeding, and this requires a thorough understanding of mechanisms of salt stress tolerance, the target sites/ defensive genes of salt stress, and signalling pathways in plants. In *Arabidopsis* plants salt tolerance was tested in wild-type and transgenic plants overexpressing *AtNHX1*. Wild-type plants displayed progressive a general growth inhibition when watered with a NaCl (0, 50, 100, 150, 200 mM) containing solution but transgenic plants were unaffected by up to 200 mM NaCl and plant development was not compromised. However, transgenic plants grown at 300 mM NaCl displayed a reduction in leaf size and chlorosis (Apse et al., 1999). So, genetic manipulation via transgenic has its limits and salt stress tolerance in plants is a multigenic trait due to which success has been rather scarce so far. The salt stress tolerance in plants is considered a multigenic trait. Lin et al. (2004) and Yamaguchi and Blumwald (2005) adds to the complexity in understanding the molecular and physiological mechanisms.

Another possible way to address the issue is ameliorating detrimental effects of salinity by exogenous application of various organic and inorganic chemicals (Farooq et al. 2009). One of these ameliorating substances is nitric oxide (NO). In the least two decades, NO has emerged as an important signalling molecule which mediates biotic and abiotic stress tolerance in plants (Molassiotis et al. 2010; Hamayun et al. 2010). Several features of NO such as its free radical status and lipophilic property aids it in diffusing through the membranes. NO readily reacts

with superoxide anions generating less toxic metabolites, metalloenzymes, and reactive oxygen species (with ROS), regulating signal pathways including those involved in seed germination, flowering, and defence response to abiotic and biotic stress (Delledonne 2005; Simpson 2005). Modulation of secondary messengers, protein kinase and plant hormones (auxin, abscisic acid, and cytokinin) communicate the NO effects in the plant. For example, NO and cGMP are downstream effectors of auxin in root organogenesis, root hair formation, and lateral root development (Pagnussat et al. 2002).

NO is known to be a key regulator of ABA and thus can modulate stomata responses and plant performance under water-limiting hyperosmotic conditions. The dependence of NO on stomatal closure under water deficit on ABA is dose dependent as at a low concentration of NO in leaf, stomatal closure is ABA-dependent and at higher concentrations of NO stomatal closure is induced by without ABA threshold accumulation (Xing et al. 2004). The role of NO as oxidant/ antioxidant is also dose- and microenvironment-dependent (Shi et al. 2007). NO stimulates multiple antioxidative enzymes (SOD, CAT, GPX, and GR) counteracting free radicals, which damage cellular membranes (Simaei et al. 2011; Kong et al. 2014). This leads to the enhancement in the tolerance of plant species treated with NO under stress conditions.

NO has been investigated to a great extent on plants, especially in ameliorating salt stress response. NO elicits tolerance response to several environmental stresses by acting as a signalling molecule (Zhao et al., 2004; Zhang et al. 2007; Zheng et al. 2009). A transient hike in NO endogenous levels has been reported in response to salt tolerance where NO is observed to modulate ion homeostasis (Zhao et al. 2007). The application of NO aided in maintaining ion homeostasis in leaves by enhancing the secretion rate of Na^+ in salt glands of *Avicennia marina* which is a halophyte. The increase in the net efflux rate of Na^+ was also reported (Chen et al. 2010). The ameliorative effect of NO on salt stress is dose dependent (Beligni & Lamattina 1999). The deleterious effect on lipids proteins and DNA was evident when a high concentration of NO was applied, most likely due to the formation of peroxynitrite (Lipton et al. 1993). The positive influence of NO in negating salinity in plants is widely reported. There are many lacunae in the knowledge on the interaction of NO with normal physiological processes of the plants that question the practicality of foliar NO application for ameliorative purposes. The time- and dose-dependence of NO application also need further studies, and the focus should be shifted from merely observational work to mechanistic explanation of the underlying processes. The present study is an attempt to investigate the efficacy of NO as a

foliar spray in managing salt stress on two cultivars of barley (salt-tolerant species) and pea (salt-sensitive species). The standardized concentration of NO donor sodium nitroprusside (SNP) was chosen to check the ameliorating effect on biomass yield, Fv/Fm, CO_2 assimilation, chlorophyll content, ion flux response, and K^+/Na^+ concentration in xylem on the glasshouse grown, as well as excised leaf segments of pea and barley cultivars. The results of this work confirm beneficial effects on elevated NO levels on plant adaptive responses to salinity but question the practicality and efficacy of this practice for growers.

Materials and methods

Plant materials and growth conditions

Barley seeds (*Hordeum vulgare* L. cv CM72 and Gairdner) were obtained from the Australian Winter Cereals Collection and multiplied in the field of TIA (Tasmanian Institute of Agriculture) facilities at Launceston, Tasmania. Pea (*Pisum sativum* L. cv. Onslow) seeds were obtained from the commercial supplier (Hollander Imports, Hobart, Australia). Seeds were surface-sterilized with commercial bleach containing 0.1% (v/v) Triton for 10 min and thoroughly rinsed with distilled water before use. Seeds were sown at 20 mm depth in a 4 L pot and thinned to 8 healthy seedlings and grown under controlled greenhouse conditions (mean temperature ranged between 19 ˚C and 26 ˚C; photoperiod 12-14 h; average humidity~65%) in the glasshouse at the School of Land and Food, University of Tasmania. The potting mixture included 70% composted pine bark, 20% course sand, 10% sphagnum peat (pH 6.0) which was fertilised (1.8 kg m^{-3} dolomite, 6.0 kg m^{-3} Osmocote Plus and 0.5 kg m^{-3} ferrous sulphate). Control plants were irrigated daily with tap water. The first fully expanded leaf was removed from the barley and pea plants grown under control conditions for 3 weeks. After excision, barley and pea leaves were placed in Petri dishes containing various concentrations of NaCl (barley- 0, 200 & 400 mM, pea- 0, 50 & 100 mM), with and without NO inducer (SNP 100 µM). Petri dishes were placed in a controlled growth room for 4 days at 23ºC air temperature with an irradiance of 150 µmol $m^{-2}s^{-1}$. For foliar spray treatment, plants were grouped according to different treatments of salt and NO inducer. After two-leaf stage plants were irrigated daily with water containing various levels of salinity (NaCl) and a foliar spray of SNP 100 µM solution containing 0.1% (v/v) triton was applied simultaneously for three weeks. To maintain the consistency foliar spray of distilled water sprayed on other plants. A randomized complete block design was used, with four replicate pots for each treatment.

Measurements of physiological parameters

Fresh weight was measured by Mettler BB2440 Delta Range balance (Mettler-Toledo, Griefensee, Switzerland) after removal of excess water by blotting shoots with paper towels. Shoots were dried at 65ºC for 2 days in Unitherm Dryer (Birmingham, UK) and weighed. Leaf sap was extracted as described by Cuin *et al.* 2009, by squeezing the first fully expanded leaf young leaves in the Eppendorf tubes then centrifuged at 7000 g for ten minutes. Cell sap osmolality was then measured by vapour pressure osmometer (Vapro, Wescor Inc. Logan, Utah, USA). 50 µl supernatant was then extracted from Eppendorf tube and diluted to 5 ml with distilled water, following by quantification of leaf sap Na^+ and K^+ content on flame photometer (PFP7, Jenway, Felsted Dunmow, Essex, England). Stomatal conductance (Gs) was measured on first fully expanded leaf using a Decagon leaf porometer (Decagon Devices Inc., WA, Australia), under constant light conditions (artificial light of 150 µmol m^{-2} s^{-1}). CO_2 assimilation (A), were measured from first fully expanded leaf using an LI-6400XT infrared gas analyser (Li-Cor Inc., Lincoln, NE, USA) in an experiment during the daytime (11 am – 2 pm). Measuring chamber was constant at a flow rate of 400 mol s^{-1}, a saturating PAR at 1500 µmol $m^{-2}s^{-1}$, a CO_2 level at 400 µmol mol^{-1}. Relative chlorophyll content (SPAD) and maximum photochemical efficiency of photosystem II (PSII; chlorophyll fluorescence Fv/Fm ratio) were measured on a first fully expanded leaf by using a Minolta Chlorophyll Meter SPAD-502 (Minolta, http://www.konicaminolta.com) and Chlorophyll Fluorometer (OS-30p, Opti-Sciences, USA), respectively. Fv/Fm measurements were taken after two hours dark adaption of plants. Xylem sap samples were collected between 10 am to 2 pm using Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA). Xylem sap was extracted by applying compressed air pressure in a chamber. 14 to 16 bar pressure was used for control plants and 25 to 35 bar pressure for salt-treated plants.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) with four replicates. Each replicate consisted of four pots, each containing eight plants. Data were

statistically analysed using analysis of variance (ANOVA) and significance difference was compared at 5 % probability level using Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) software. Graphs were made using Microsoft Excel, v 2010, and error bars in the graph represented standard error of four replications per treatment. Means sharing a common letter in the column were not significantly different at 5% probability. Error bars represented SE.

Results

In order to assess the palliative effect of NO on salt-induced stress response of two barley cultivars, CM 72 (salt-tolerant) and Gairdner (salt-sensitive); see Chen et al. 2007 for quantitative yield data), *in vivo* experiments have been carried out on intact plants grown in greenhouse under salt stress and *in vitro* experiments on leaf strips harvested from normal plants and exposed to salt stress of chosen concentrations for set period of the experimental time. In our early trials which are not reported here, higher concentrations (< 500 µM) were sprayed on intact barley and pea plants to tackle the issue of NO diffusion into leaf tissue but the effect wasn't significantly visible. However, in potato leaf high SNP concentration (1 mM) was found to be toxic itself, as a great accumulation of malonyldialdehyde (MDA) occurred (Beligni & Lamattina 2002). Lipid peroxidation is one of the first consequences of oxidative damage, MDA production was used as an indicator of oxidative stress (Halliwell & Gutteridge 1984). To see the dose dependency of NO on salt tolerance, the leaf segment of barley cultivar -Gairdner was subjected to 100 mM NaCl and treated afterwards with varying concentration of SNP (20, 50, 100, 200, 500 µM). The positive effect of NO donor- SNP was prominent ($P< 0.05$) on the chlorophyll content at 100 µM concentration (Fig 1). This concentration of SNP was selected as optimal, and further experiments were conducted with the same. The leaf segments of Gairdner, CM72, and pea were treated with 50 & 100 mM of NaCl and later 100 µM SNP was applied to see the effect on the chlorophyll content of leaf segments. The leaf segment of Gairdner, when subjected to NaCl treatment, experienced 50% reduction in the chlorophyll content. The results indicate the significant enhancement ($P< 0.05$) in the chlorophyll content on application of SNP in leaf segments of both barley cultivars and pea though the levels were lower than the control (Fig 2.1 to 2.5).

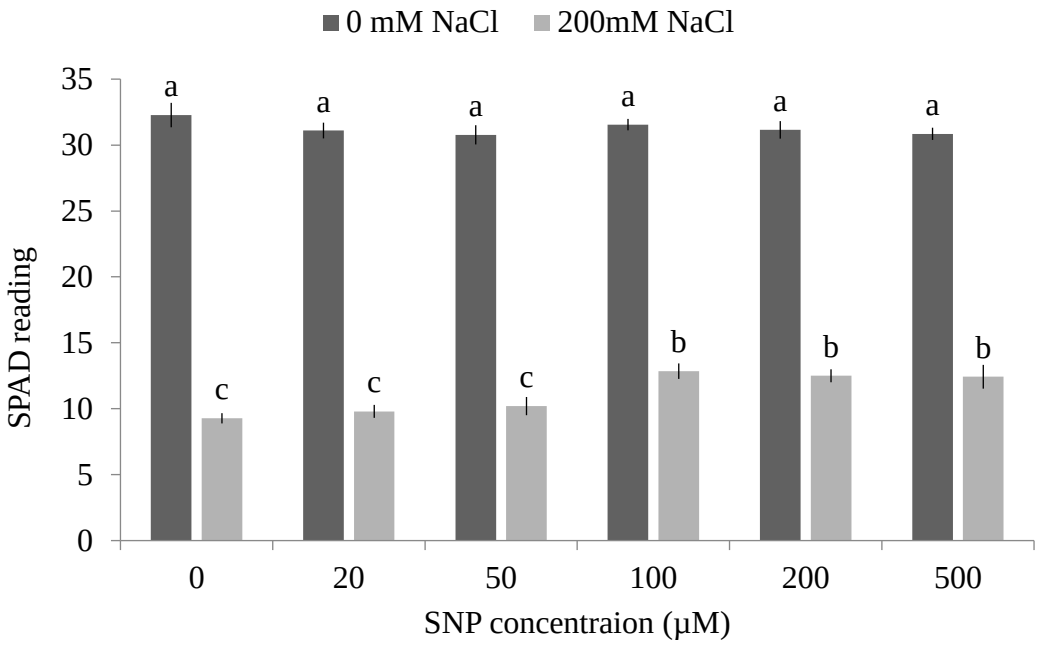

Figure 1-: Effect on the chlorophyll content SPAD of barley (cv Gairdner) leaf segments placed into a Petri dishes under two levels of salinity (NaCl). To find optimum SNP concentration various concentrations of SNP have been added to petri dishes media for four days until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

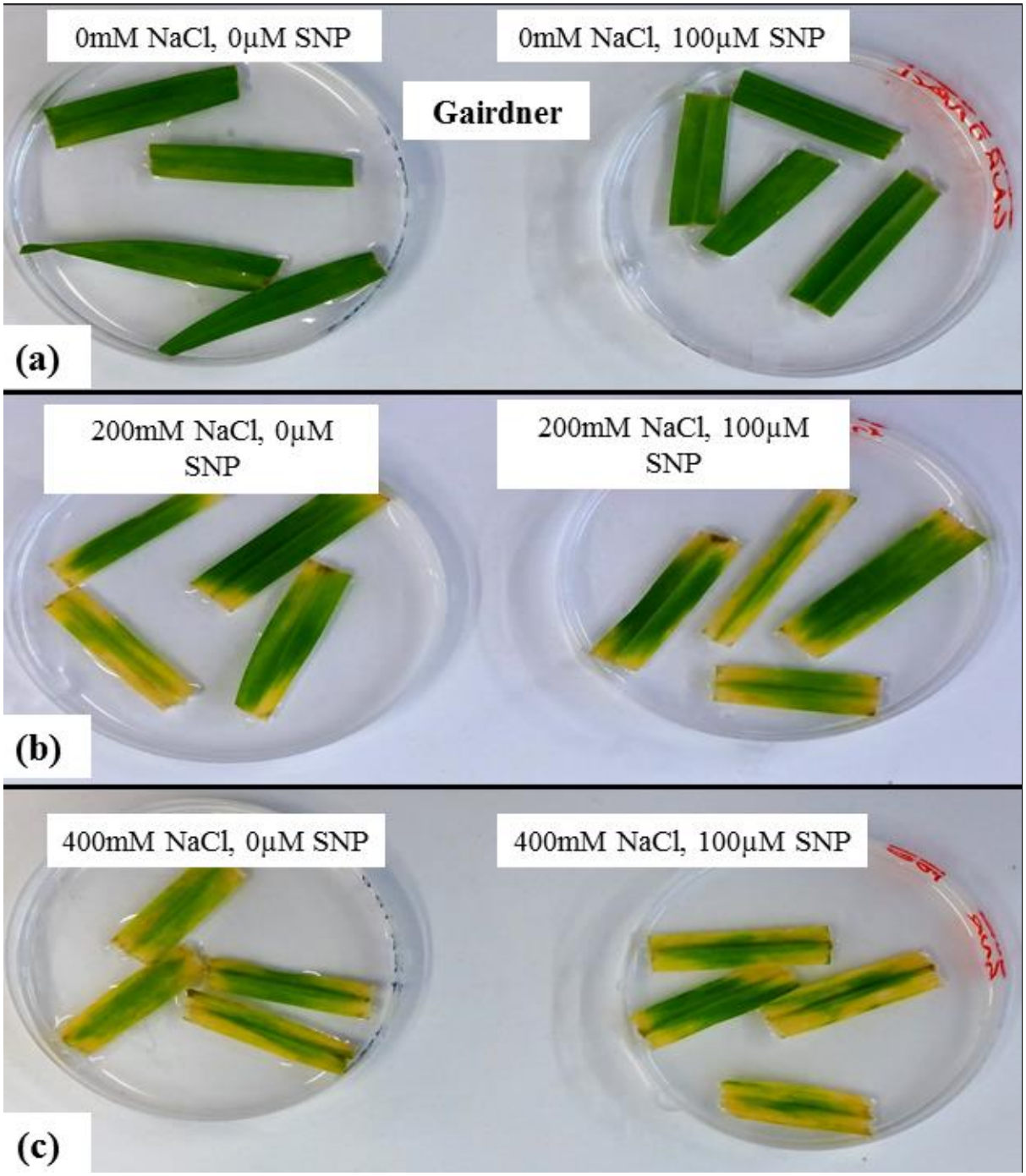

Figure 2.1-: Effect on the chlorophyll content SPAD of barley (cv Gairdner) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until images were taken. Means ± SE (n = 8).

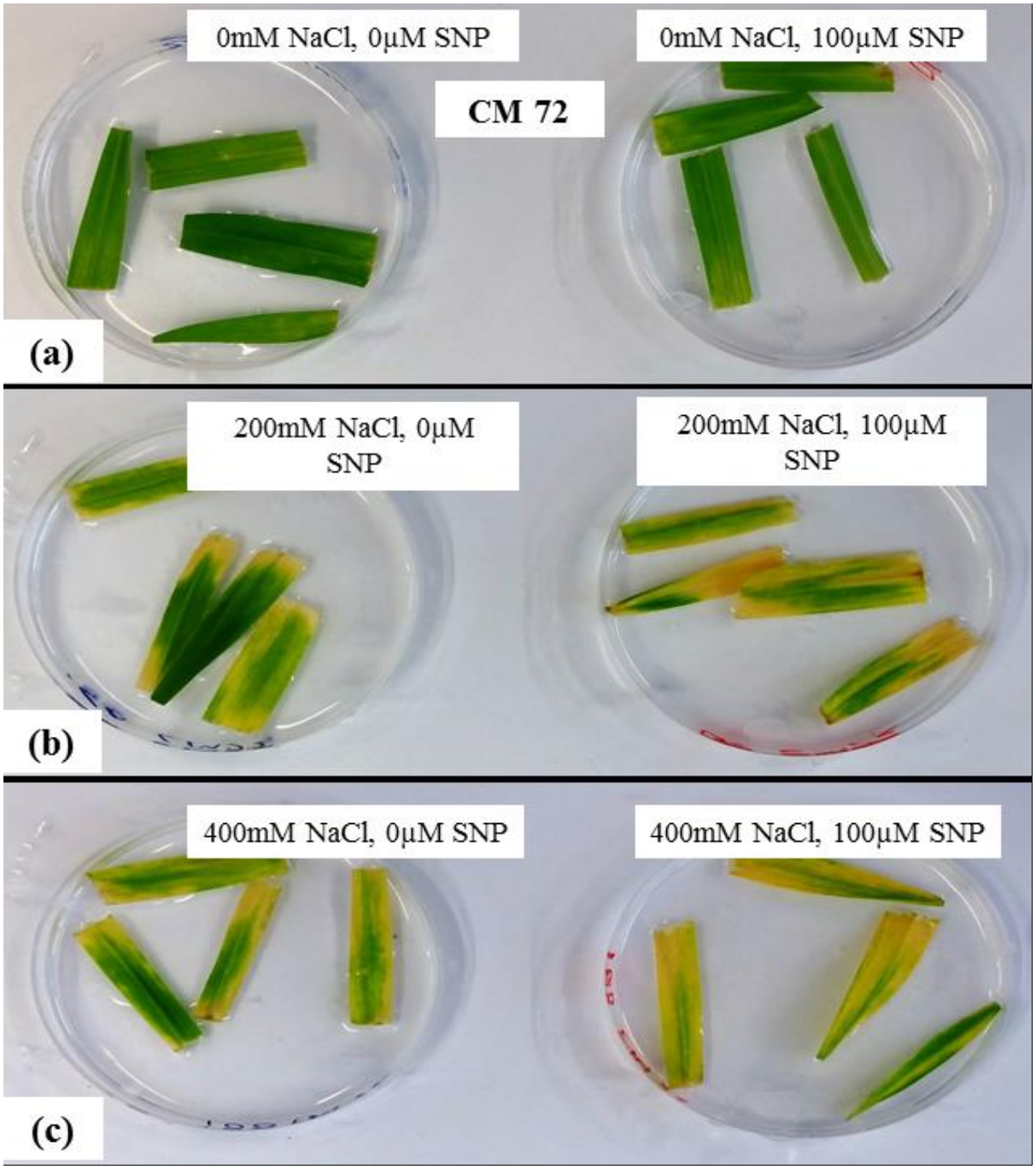

Figure 2.2-: Effect on the chlorophyll content SPAD of barley (cv CM 72) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until images were taken. Means ± SE (n = 8).

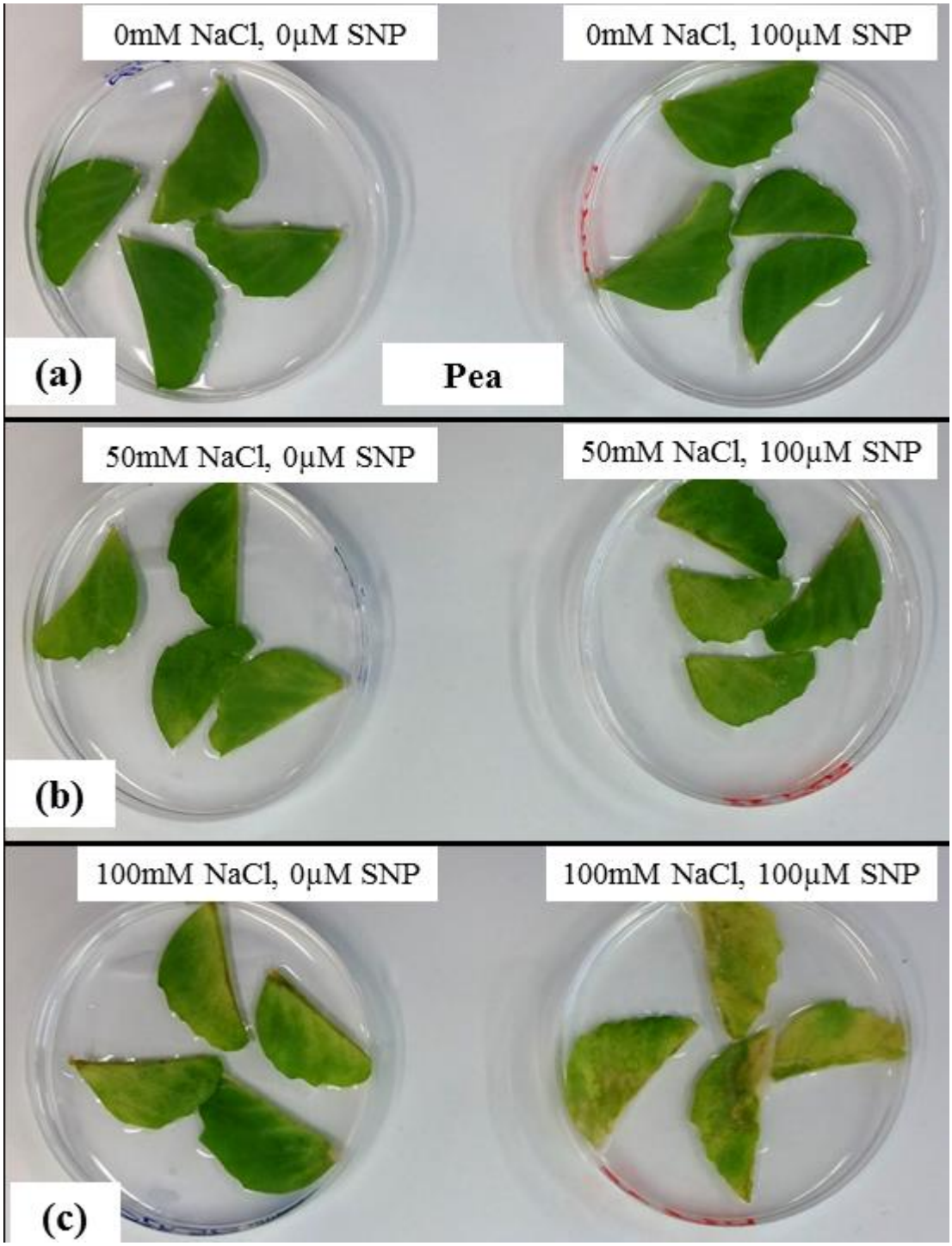

Figure 2.3-: Effect on the chlorophyll content SPAD of pea (cv Onslow) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until images were taken. Means ± SE (n = 8).

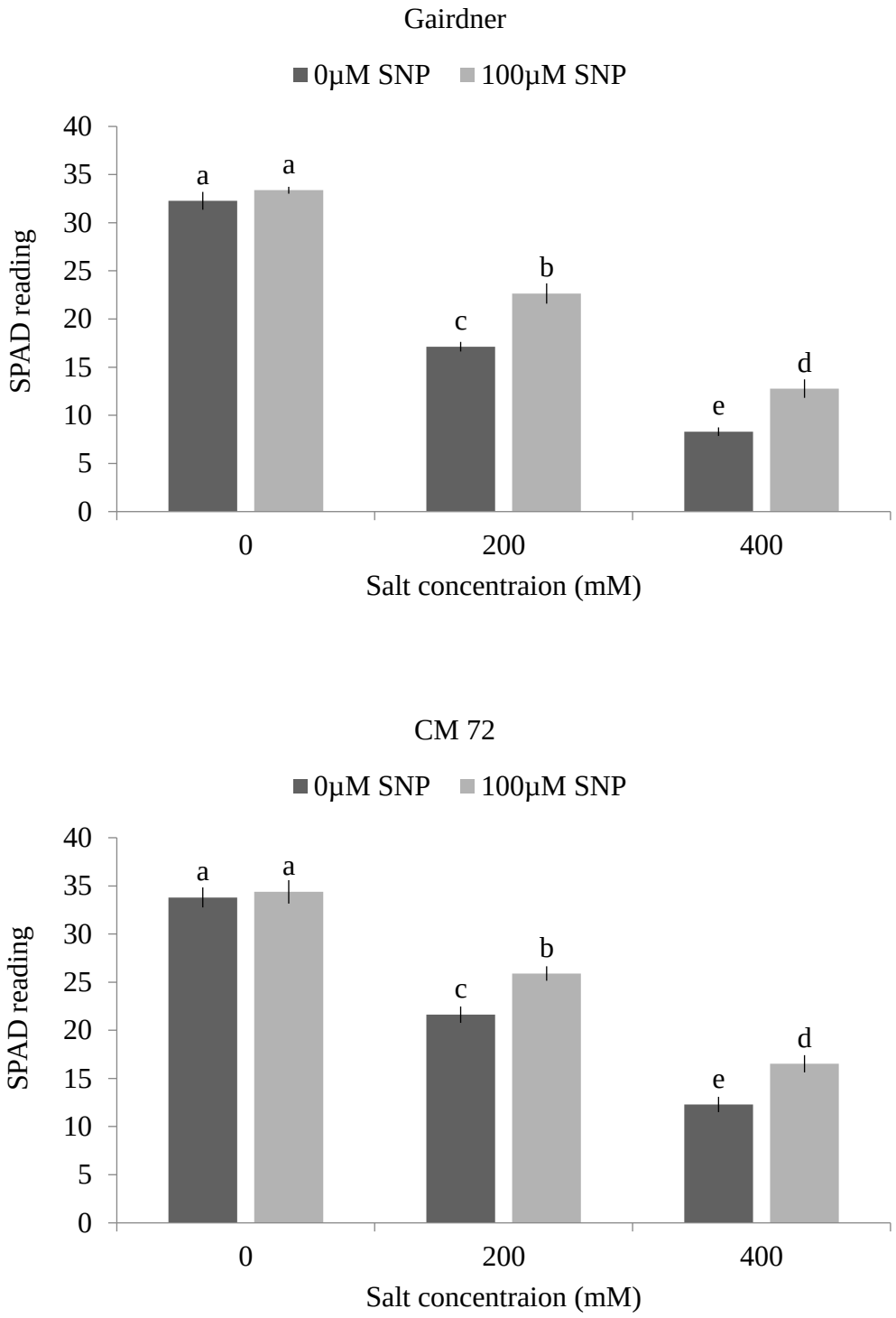

Figure 2.4-: Effect on the chlorophyll content SPAD of barley (cv Gairdner and CM 72) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

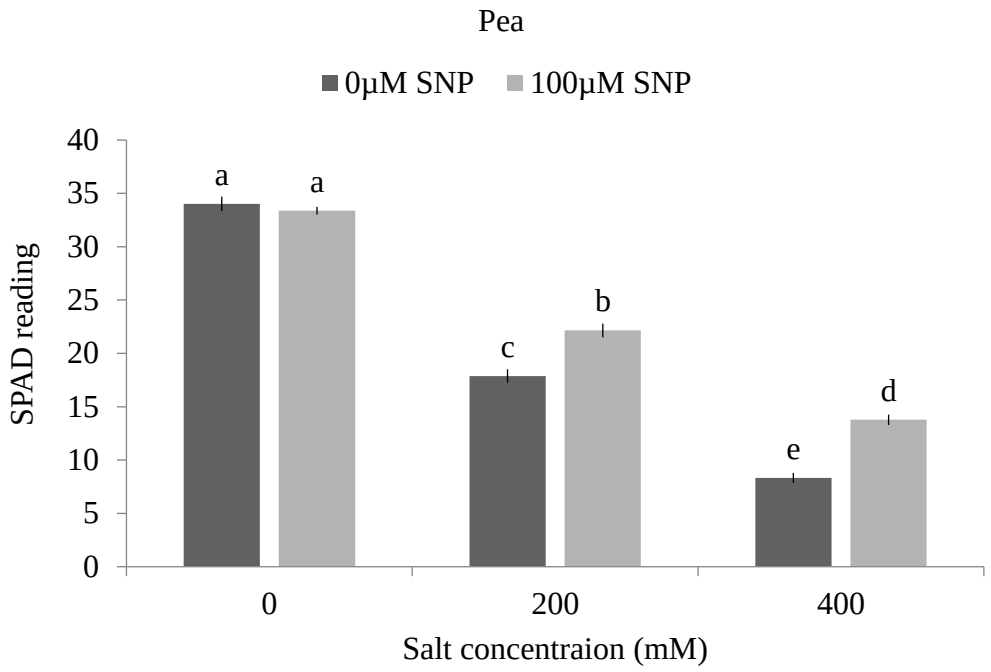

Figure 2.5-: Effect on the chlorophyll content SPAD of pea (cv Onslow) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

Photochemical efficiency is a significant parameter that can indicate the positive/negative impact of the entity on the plant growth and development. NaCl treatment of 50 mM & 100 mM for both barley cultivars and 25 mM & 50 mM for pea reduced the photochemical efficiency drastically (Fig 3.1 & 3.2). SNP treatment (100 µM) had a positive influence on the photochemical efficiency of both the cultivars of barley and pea. It is apparent that the positive influence of NO come into effect as soon as salt stress sets in, thus enhancing photochemical efficiency and chlorophyll content index in both the cultivars of barley as well as in the pea. The ameliorating effect of NO on excised leaf segments points to the probability of restriction or delay in entry of NO into the mesophyll when applied to the intact plant.

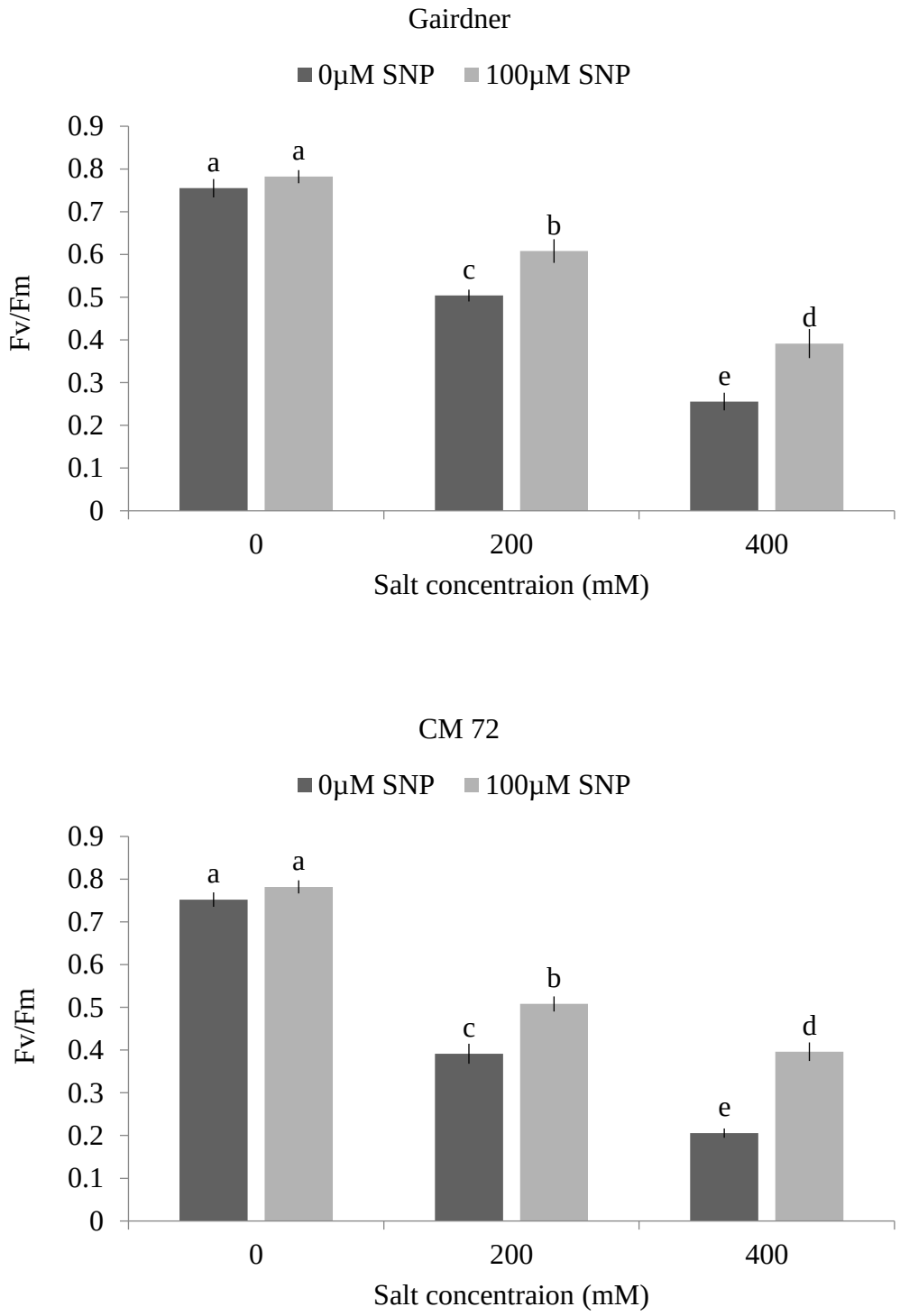

Figure 3.1-: Effect on the photochemical efficiency Fv/Fm of barley (cv Gairdner and CM 72) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

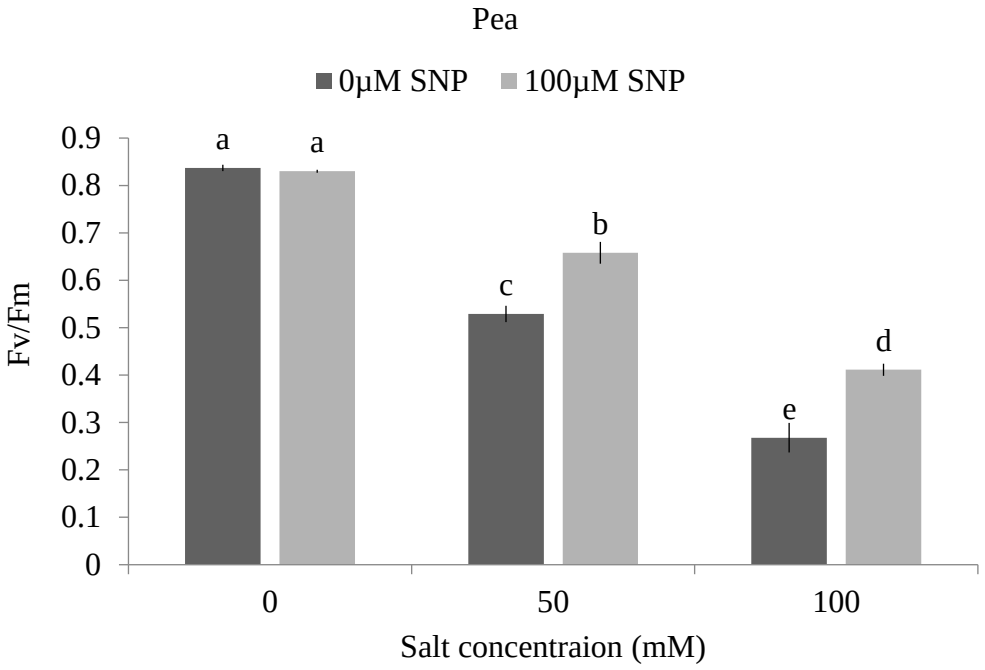

Figure 3.2-: Effect on the photochemical efficiency Fv/Fm of pea (cv Onslow) leaf segments placed into a Petri dishes under various levels of salinity (NaCl) & SNP for four days until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

We then tested the applicability of exogenous NO application as an ameliorating agent for pot-grown plants in the glasshouse. The salt stress treatment for three weeks had negative impact on the growth and biomass of both cultivars of barley plants, resulting in a significant ($P < 0.05$) reduction in the fresh and dry weights of the plants. The increase in the concentration of NaCl proportionately lowered the biomass accumulation as can be observed in the extent of reduction of fresh and dry weights of the plants at 200 mM and 400 mM of NaCl respectively (Fig 4.1 & 5.1). A drastic reduction in the biomass was observed in both fresh and dry weights of the plants ($P < 0.05$) under salt stress, while the extent of biomass reduction was more pronounced in Gairdner than in CM 72 under salt stress. At the same time, SNP (100 µM) application did not show any ameliorative effects on biomass in either cultivar of barley (Gairdner & CM72) plants under salt stress (Fig 4.1 & 5.1). The same effect of NaCl was evident in the fresh and dry weight of pea (Fig. 4.2 & 5.2). The foliar spray of 100 µM SNP failed to show any significant positive influence on the biomass yield of a pea plant.

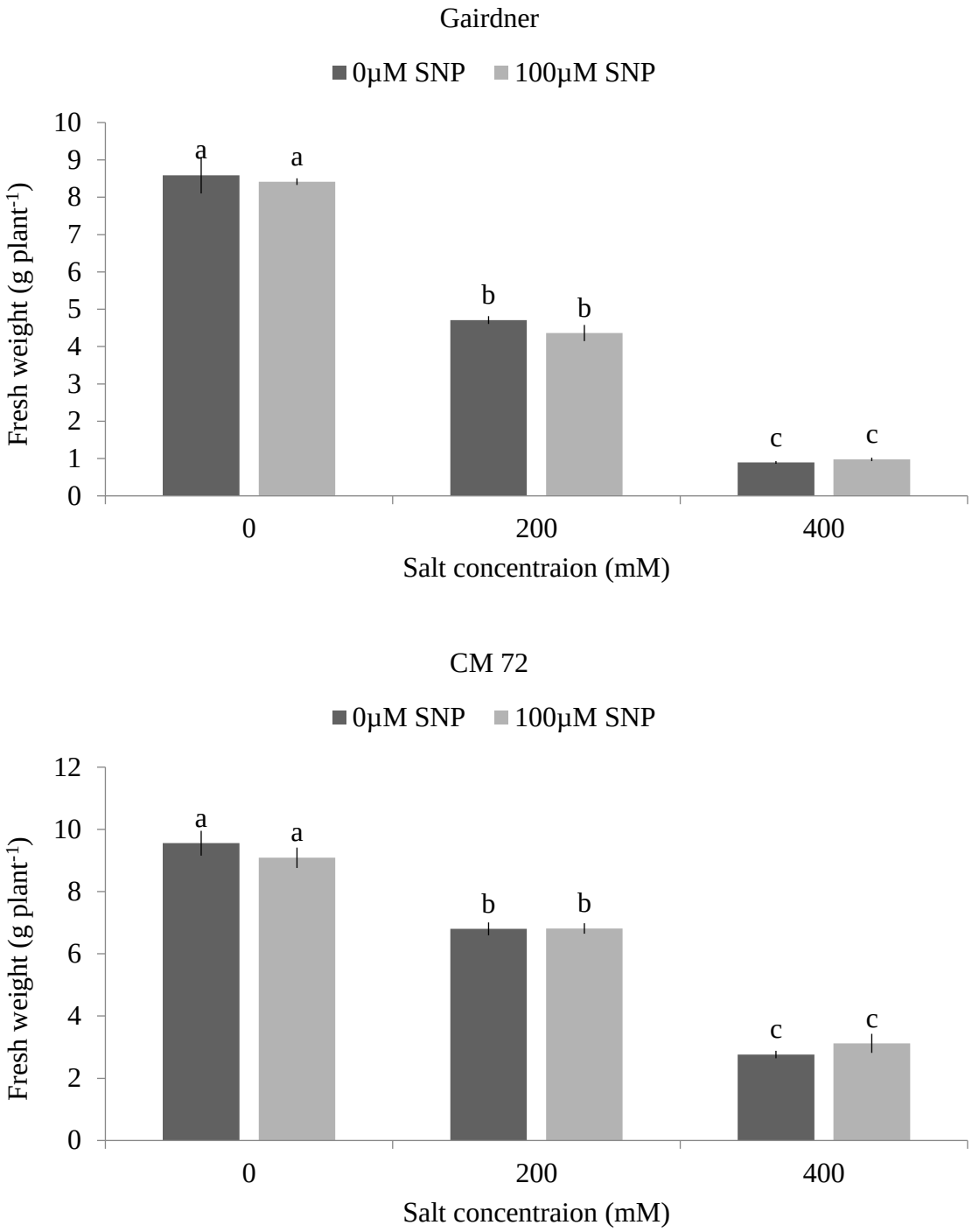

Figure 4.1 -: Effect on the fresh shoot weight of barley (cv Gairdner and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

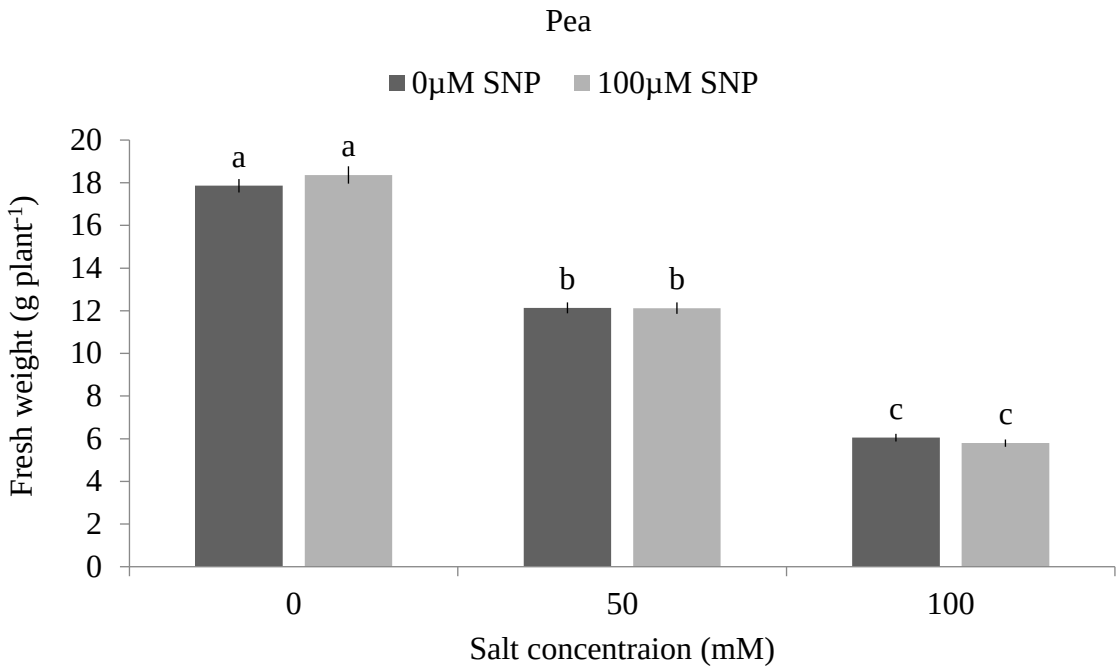

Figure 4.2 -: Effect on the fresh shoot weight of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

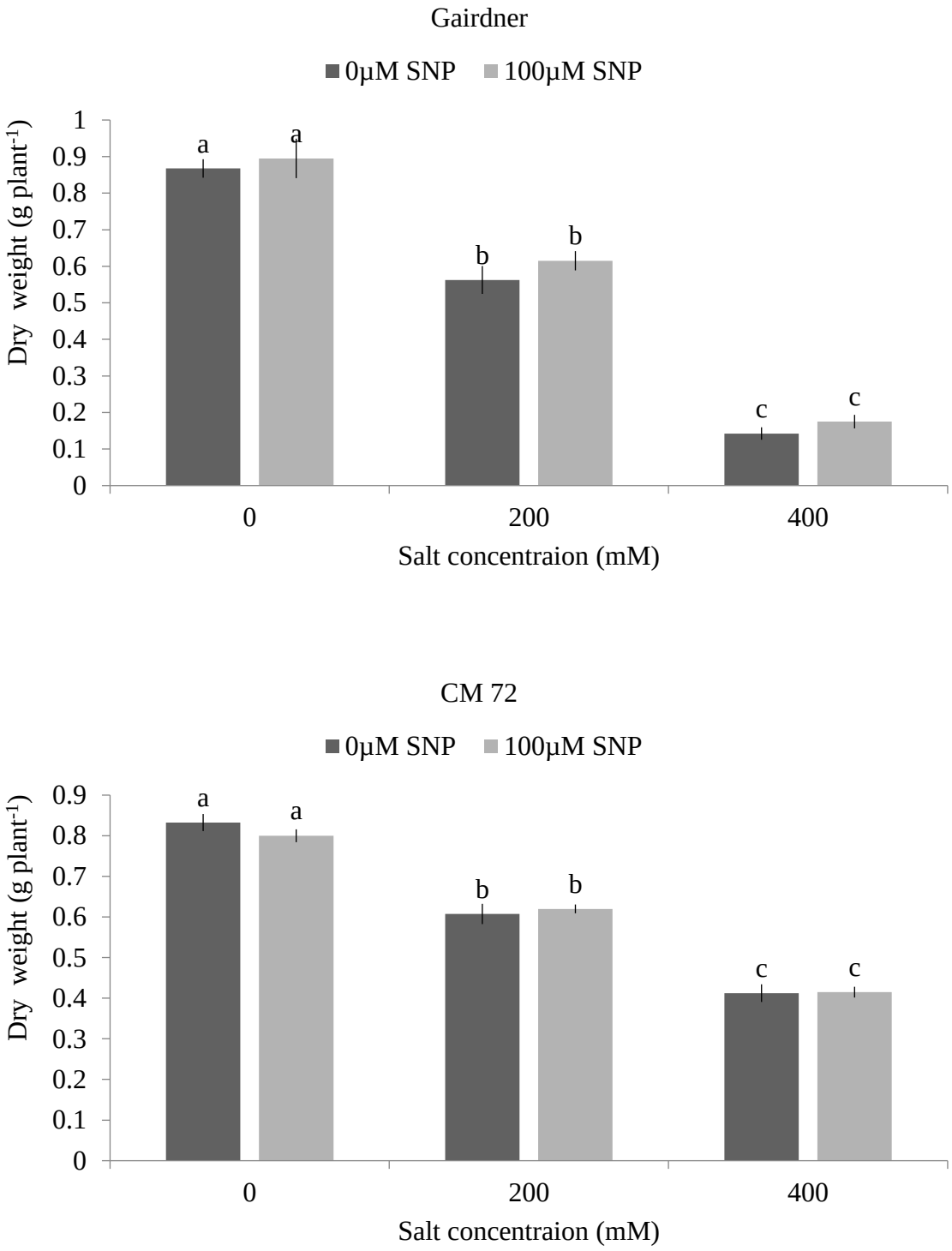

Figure 5.1-: Effect on the dry shoot weight of barley (cv Gairdner and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

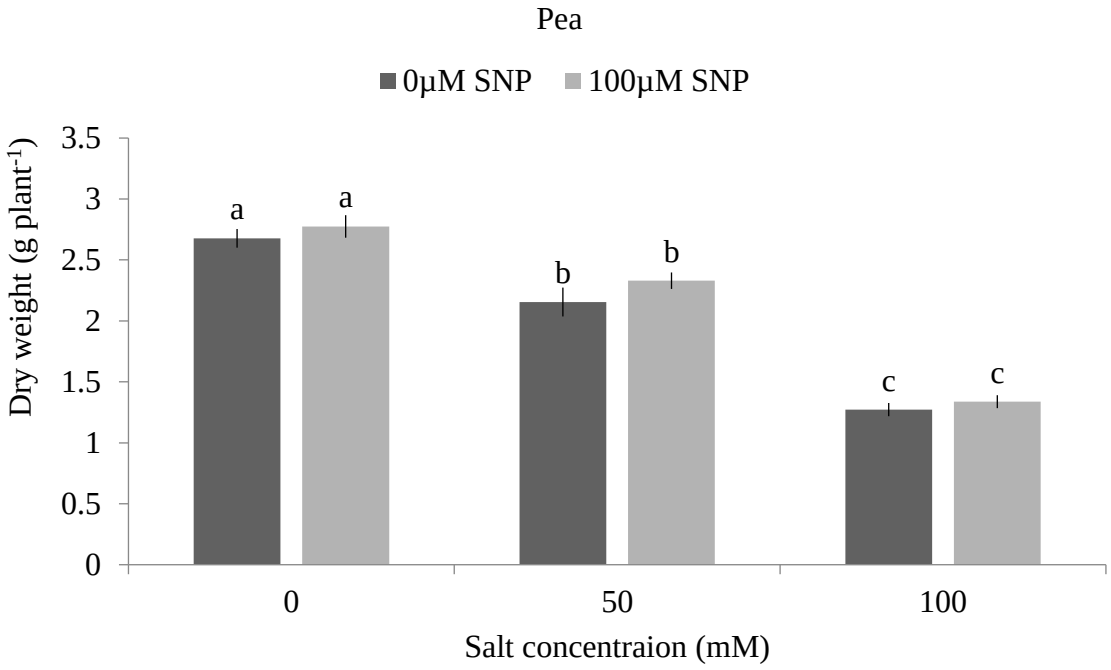

Figure 5.2-: Effect on the dry shoot weight of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

The photochemical efficiency was found to be inversely related to the salt concentrations applied in both the cultivars of barley (Fig 6.1). The extent of reduction in the Fv/Fm ratio was approximately same in both the cultivars. Interestingly, a trend of revival in the Fv/Fm value was observed in samples exposed to 100 µM SNP spray at a higher salt concentration (400 mM) in both Gairdner and CM 72 barley cultivars. A significant reduction in the photochemical efficiency was obvious in the pea plants (Fig 6.2) subjected to salt stress (NaCl 50 mM and 100 mM). Foliar spray of SNP (100 µM) did not show any significant positive impact in pea on ameliorating the situation.

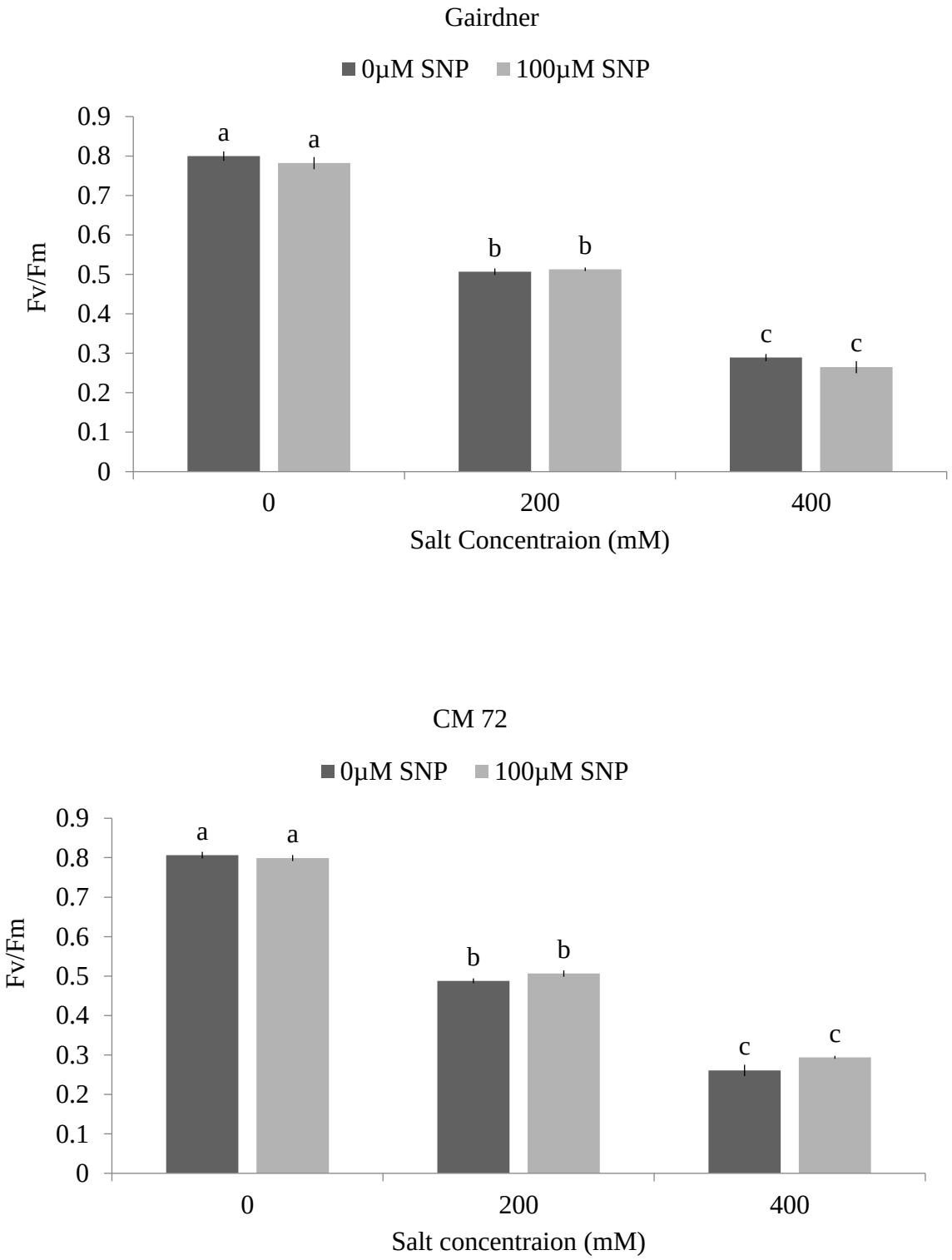

Figure 6.1-: Effect on the photochemical efficiency Fv/Fm of barley (cv Gairdner and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

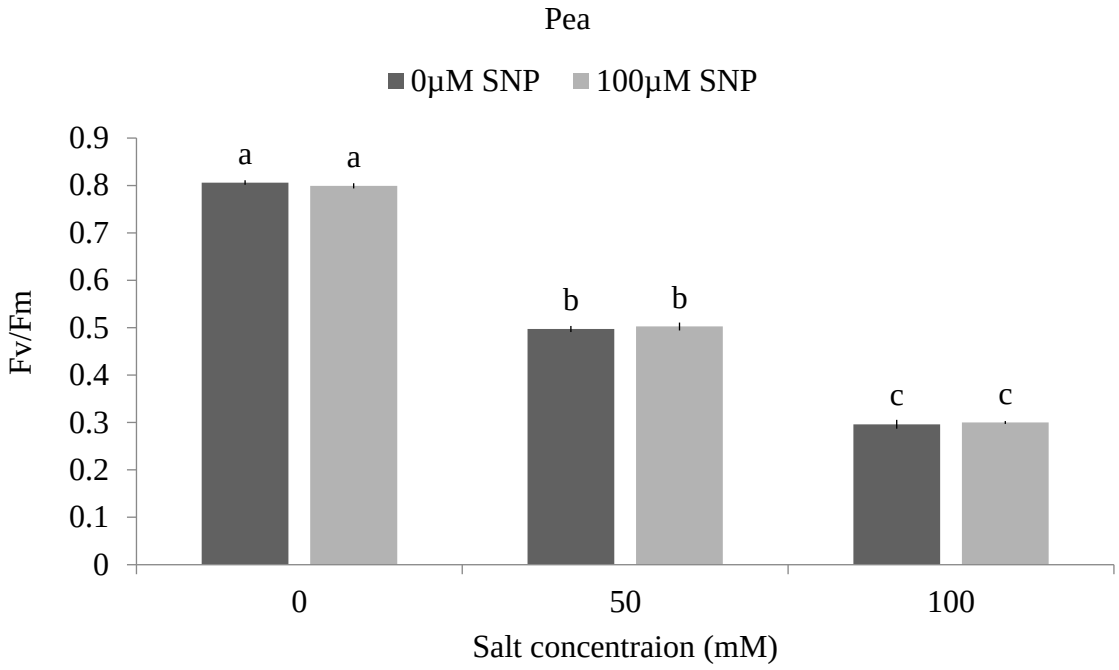

Figure 6.2-: Effect on photochemical efficiency Fv/Fm of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

Chlorophyll content index also showed an inverse trend with salt concentrations in both of the barley cultivars (Fig 7.1). However, spray treatment of SNP did not ameliorate chlorophyll content at either level of salt stress applied. Chlorophyll content of the pea plants also exhibited significant reduction under salt stress (50 mM and 100 mM NaCl), and the application of SNP spray (100 µM) did not improve the chlorophyll contents to a significant extent (Fig 7.2).

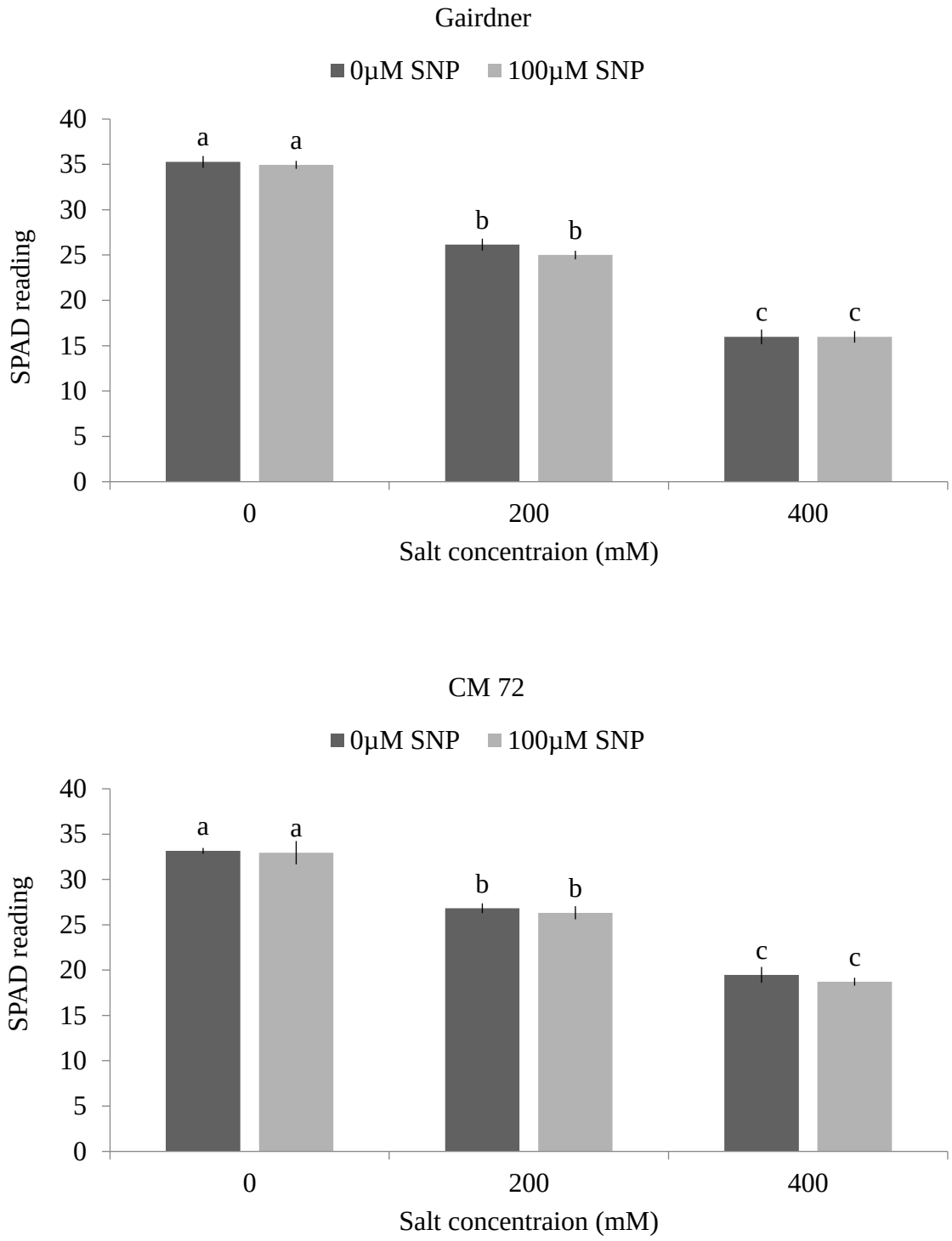

Figure 7.1-: Effect on the chlorophyll content SPAD reading of barley (cv Gairdner and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

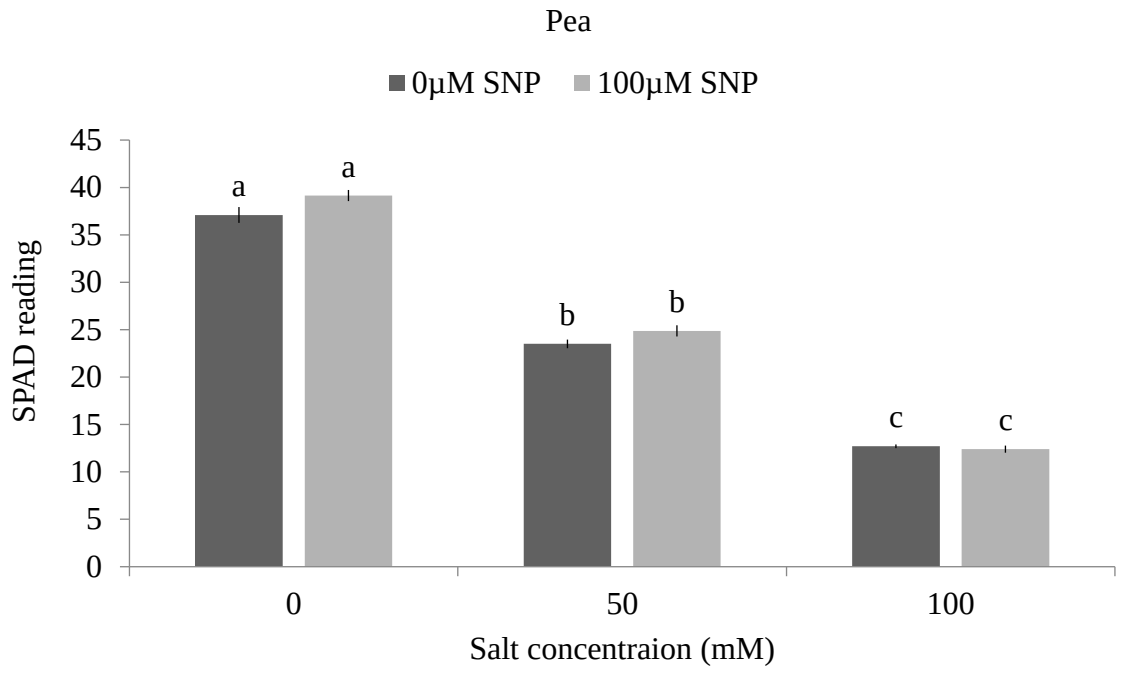

Figure 7.2-: Effect on the chlorophyll content SPAD reading of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

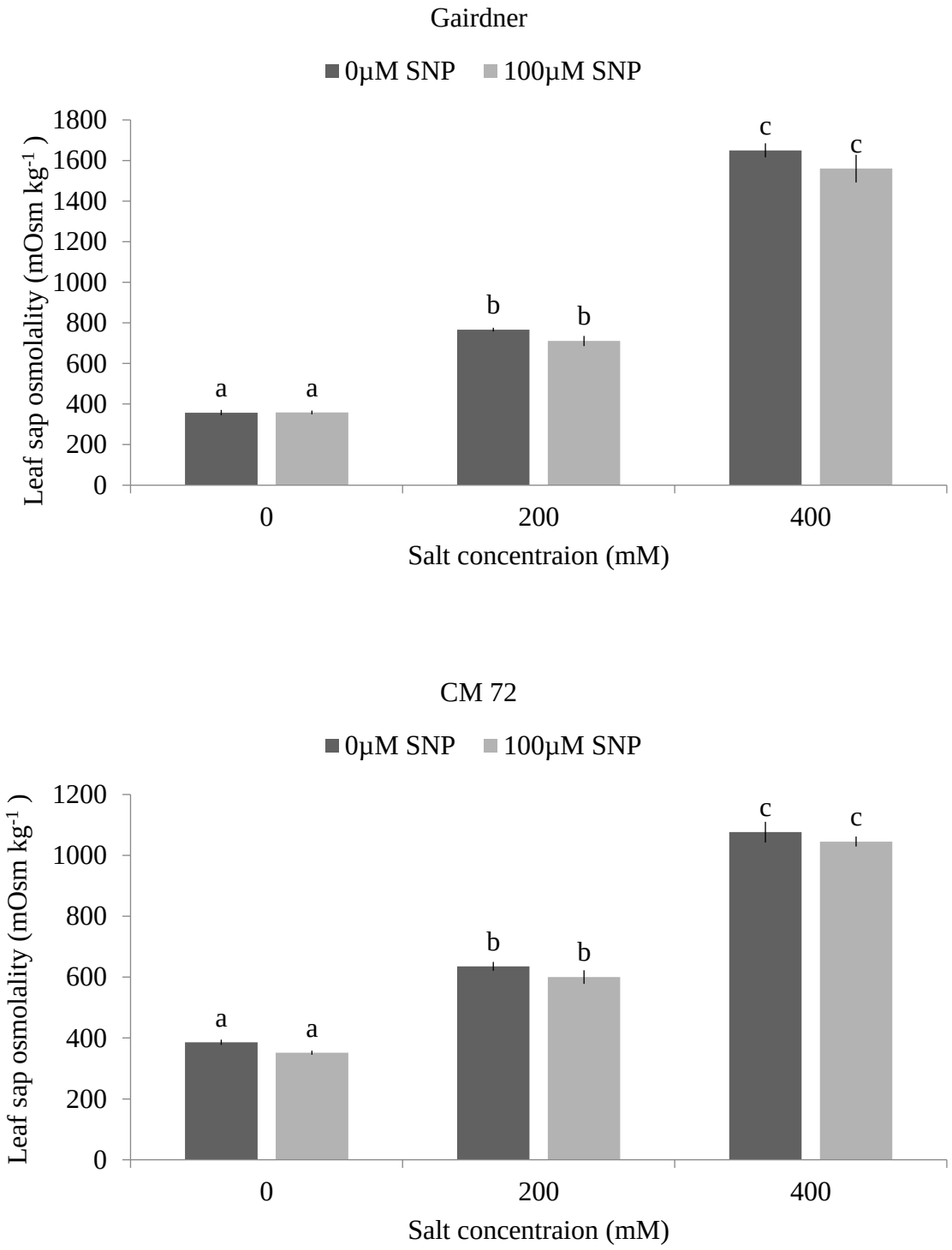

Figure 8-: Effect on the leaf sap osmolality of barley (cv Gairdner and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

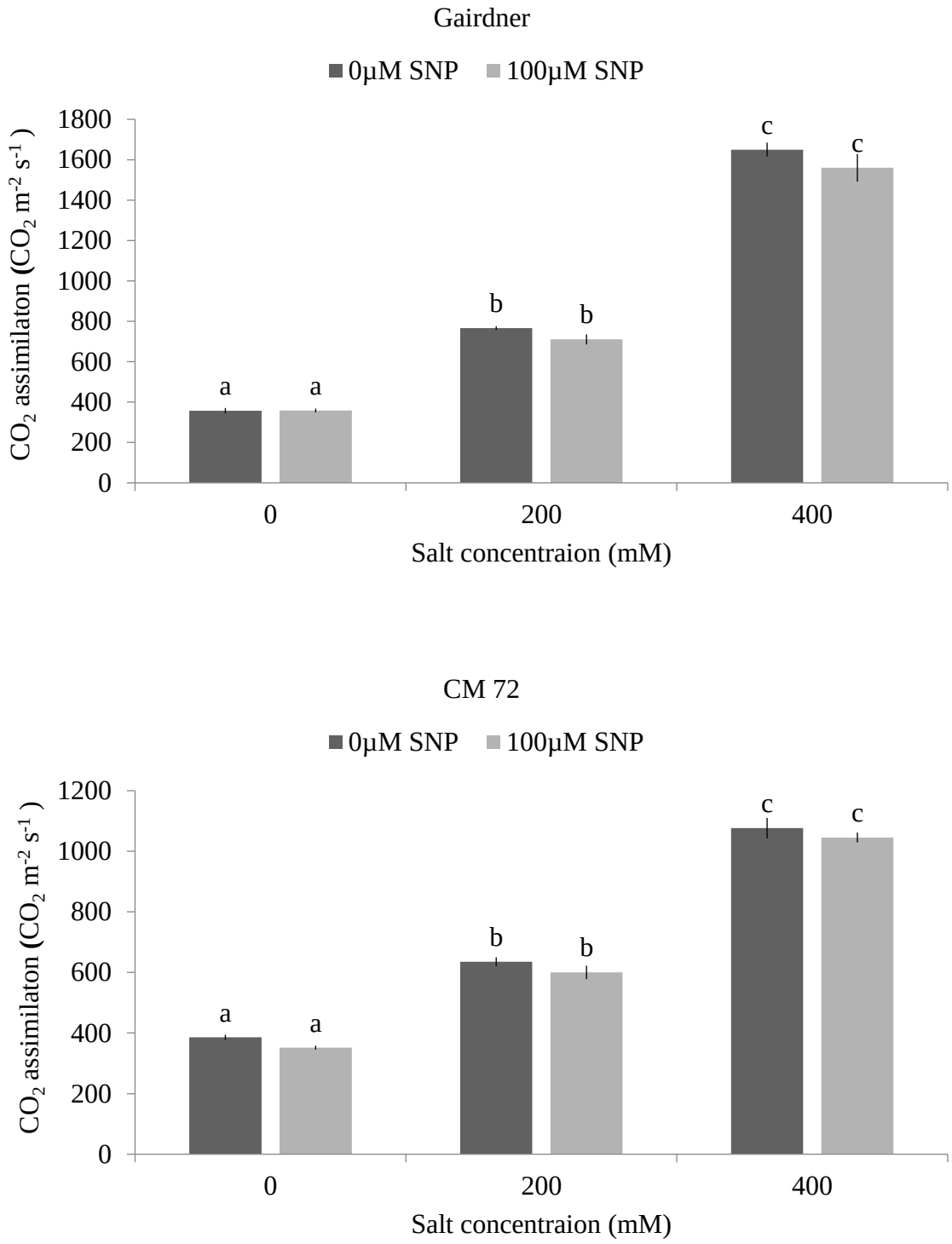

Figure 9-: Effect on CO_2 assimilation of barley (cv Gairdner and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 8). Data with the different low case letters is statistically different at $P < 0.05$

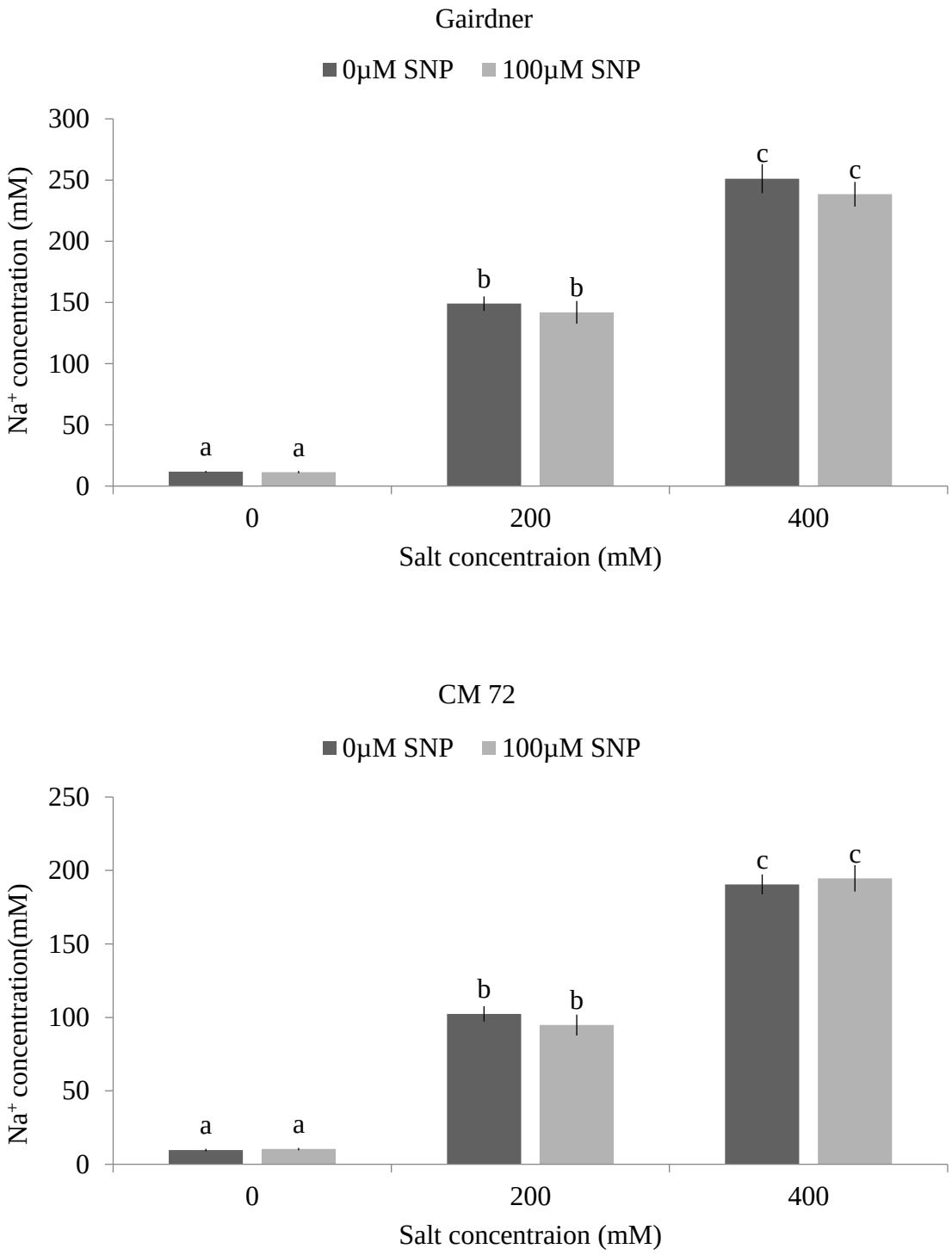

Figure 10-: Effect on Na^+ ion leaf sap concentration of barley (cv Gairden and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

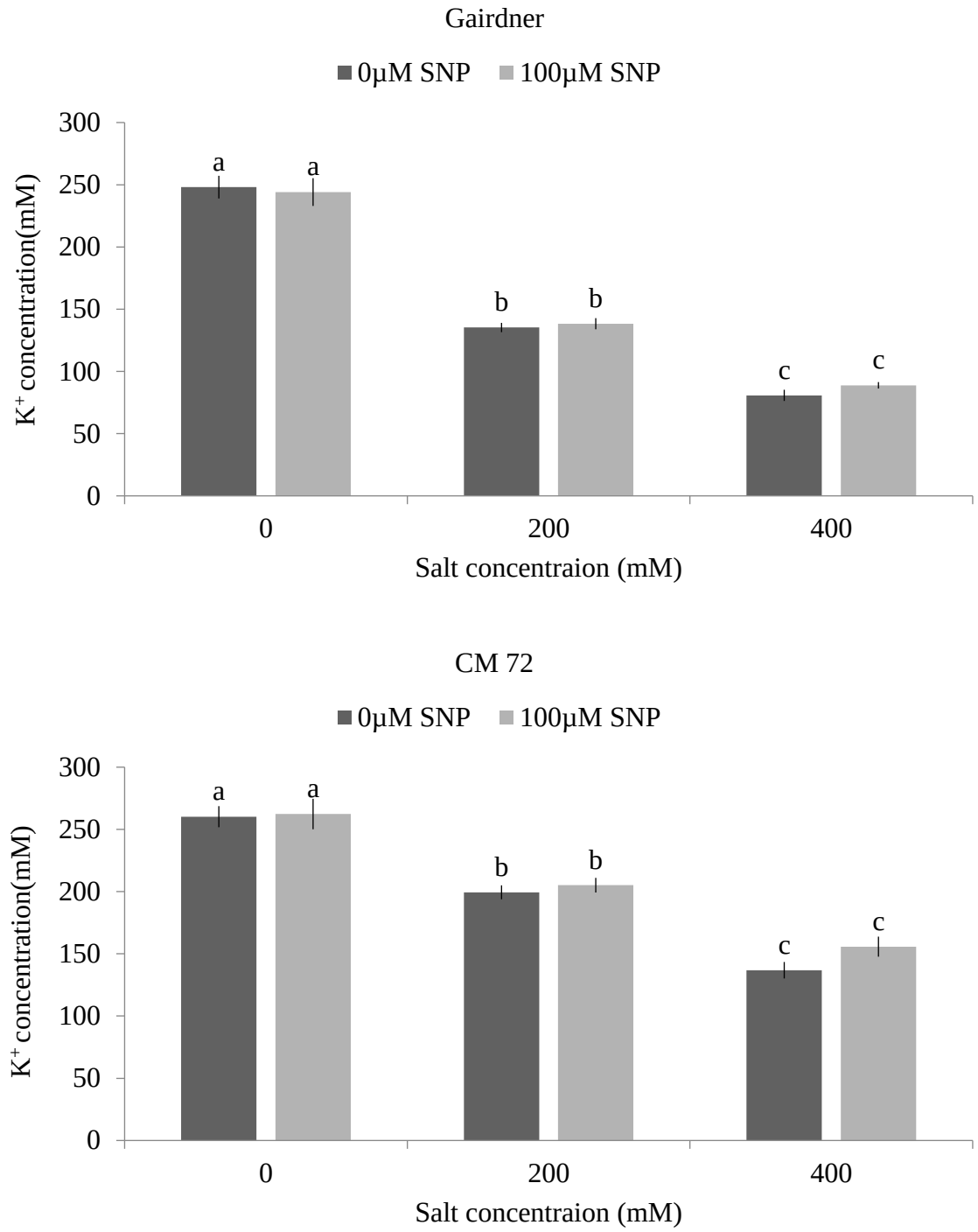

Figure 11-: Effect on K^+ ion leaf sap concentration of barley (cv Gairden and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

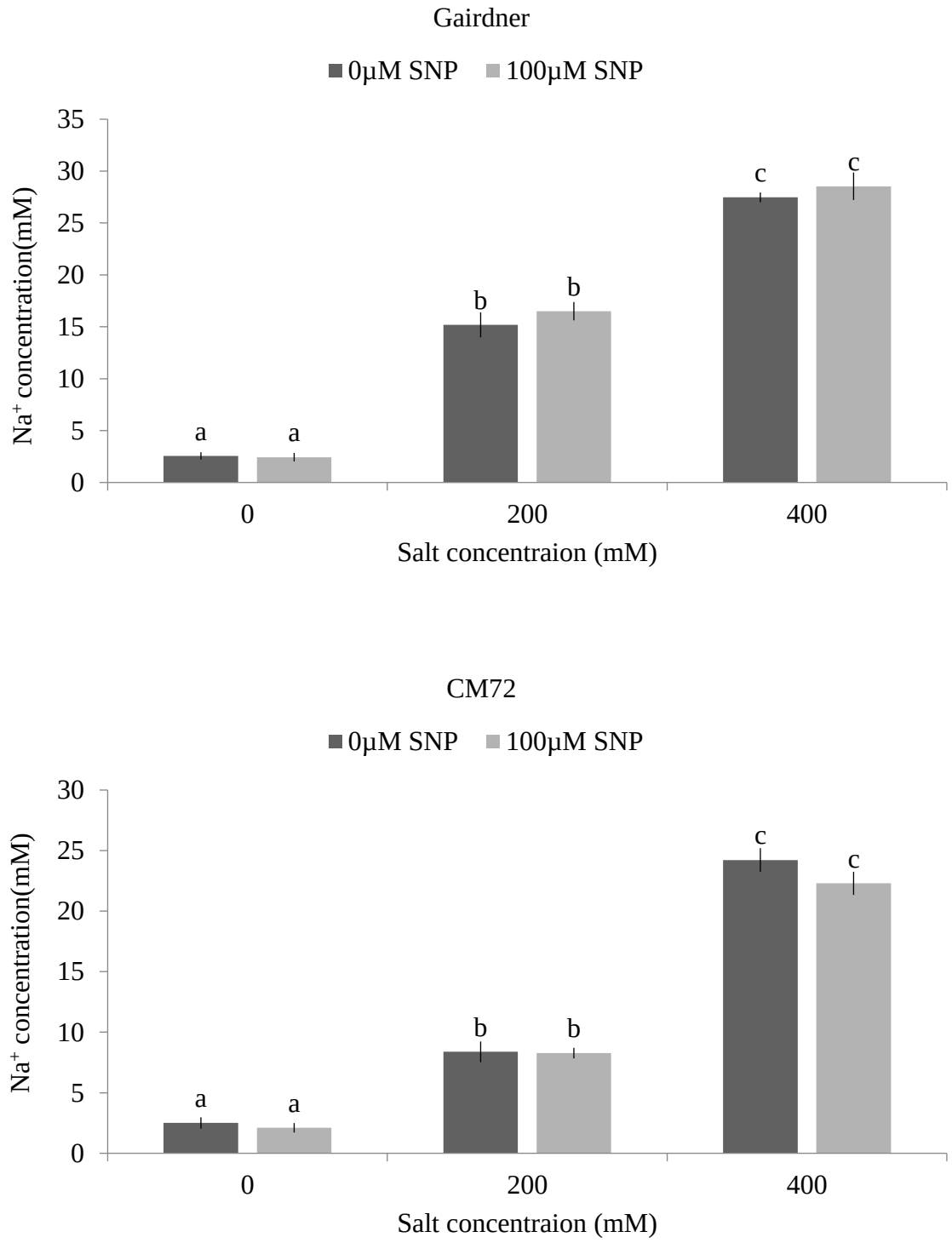

Figure 12-: Effect on Na^+ ion xylem sap concentration of barley (cv Gairden and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

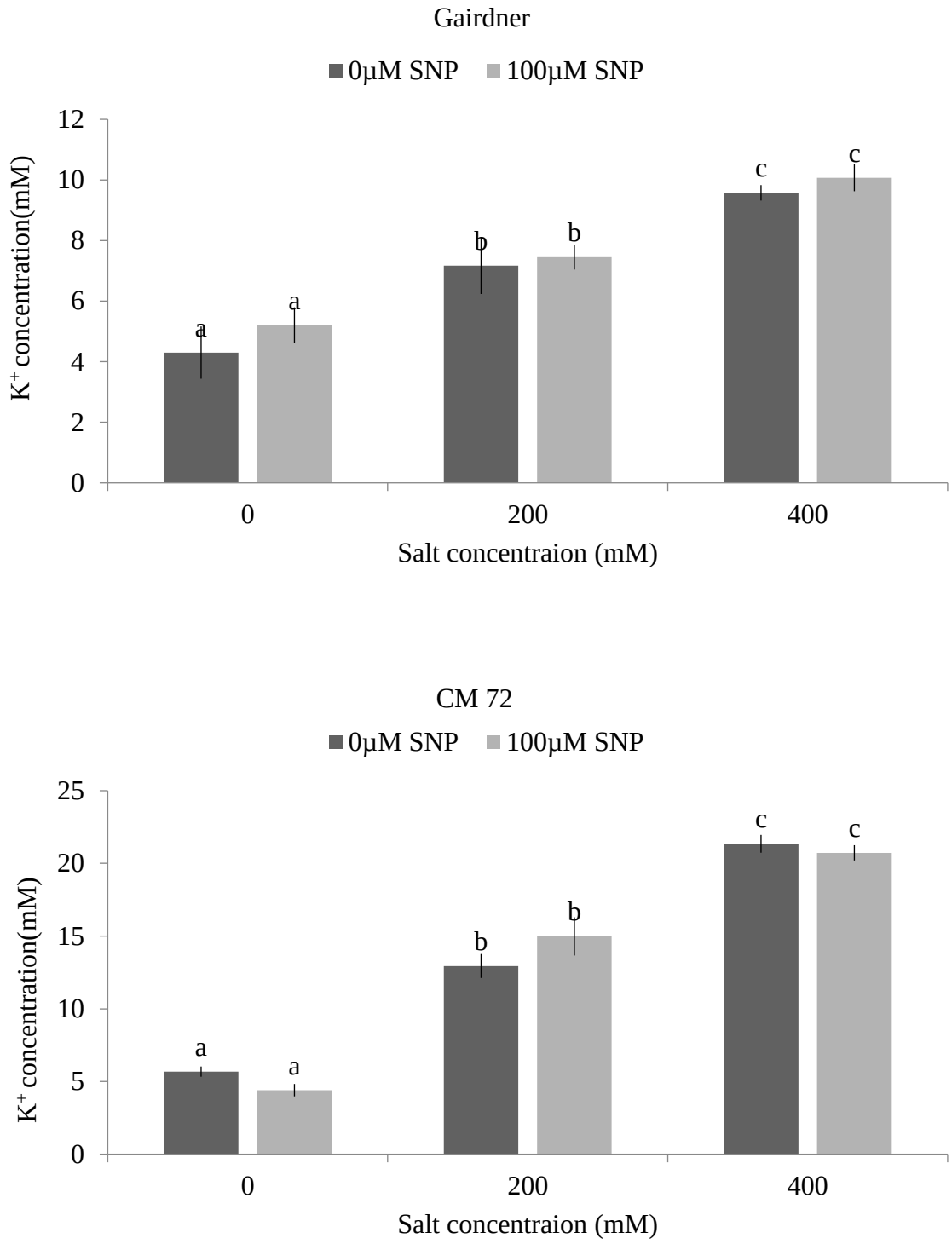

Figure 13-: Effect on K^+ ion xylem sap concentration of barley (cv Gairden and CM 72) plants grown for four weeks in glass house under various levels of salinity (NaCl) & SNP (three weeks) until measurements were taken. Means ± SE (n = 8). Data with the different low case letters is statistically different at $P < 0.05$

The leaf sap osmolality was found to increase along with an increase in the level of salt stress in both the cultivars of barley and pea evaluated (Fig 8) but influence of SNP (100 µM) spray was not significant. Salt stress significantly reduced the net CO_2 assimilation rate ($P < 0.05$) in both barley cultivars (Fig 9). An inverse relation was observed between salt concentrations and CO_2 assimilation. Meanwhile, NO (SNP spray) failed to restore CO_2 assimilation levels to any significant extent in both the cultivars. The concentration of Na^+ in the leaf sap was found directly proportional to the concentration of NaCl applied to the plants and the quantitative trend appeared almost similar in both tested barley cultivars (Fig 10). However, a spray of SNP (100 µM) could not bring a significant corrective impact towards normalizing the leaf sap Na^+ concentration. The leaf sap concentration of K^+ exhibited an opposite trend to that of Na^+, showing a decline with increasing levels of salt stress induced (Fig 11). SNP application (100 µM) was also found to be ineffective in improving the situation. Xylem sap concentration of ions acts as a crucial indicator of abiotic stress response in plants (Dodd et al. 1996). The elevation of Na^+ and K^+ concentrations in the xylem sap, observed in the barley cultivars of current study under salt stress is illustrated in Fig. 12 and 13 respectively. Application of SNP (100 µM), however, did not make a significant contribution towards the restoration of the concentrations of these ions in the xylem sap to their normal levels. A comparison of the effects of NO (applied as SNP spray) on biomass production, and photochemistry of the leaves in barley (salt tolerant plant) and pea (salt sensitive plant) from the above data apparently show similar outcomes. A significant positive impact of NO could not be achieved in both plants.

Discussion

In this experiment, SNP was used as an NO donor to study the interactive effect of NO and salt stress in two cultivars of barley, CM 72 and Gairdner, which differ in their salt stress tolerance alongside with pea, a salt-sensitive plant species. The plants were exposed to salt stress, and its effect on different physiological parameters was studied. The negative effect of salt stress was evident on the dry /fresh weight, CO_2 assimilation, chlorophyll content, and photochemical efficiency of the plants. The standardized optimal concentration (100 µM SNP) of NO donor was applied to the cultivars, and the effect was recorded. Previous studies involving NO foliar application on soybean (Zhang et al. 2004), rice (Farooq et al. 2009) and maize (Wu et al. 2011) have shown increased plant growth under salt stress conditions. However, in our study, NO failed to induce any positive effect on barley cultivars except for

photochemical efficiency, which was enhanced with the application of SNP at 400 mM salinity (NaCl) treatment. The up-regulation of the antioxidant enzyme activities is considered one of the reasons for the amelioration effect of NO. The exogenous application of SNP has been cited to eliminate oxidative stress in *K. virginica* incurred due to salt stress. The activities of catalase, peroxidase, and superoxide dismutase have been much higher after SNP spray (Guo et al. 2009).

Chen et al. (2010) experimentally proved the pivotal role of NO in achieving lower Na^+/ K^+ ratio in the cytosol by introducing various concentrations of SNP in the mangrove plant *Avicenna marina*. The optimal concentration of SNP enhanced Na^+ extrusion into salt glands by activating HA1 and SOS1 (PM- H^+-ATPase) genes, along with increased vacuolar Na^+ sequestration by stimulating VHA-c1 and NHX1 genes. However, in the current investigation, no significant effect on Na^+/ K^+ ratio was observed.

The salt tolerant CM 72 cultivar of barley exhibited higher K^+ concentrations in xylem and mesophyll cells than salt sensitive Gairdner. In contrast to this, accumulation of Na^+ and Cl^- ions in leaf sap posing ion toxicity is less in CM 72 in comparison to Gairdner maintaining high K^+/ Na^+ ratio. The hallmark feature of salt tolerance is the maintenance of high shoot K^+/Na^+ ratio (Dasgan et al. 2002; Ren et al. 2005). Earlier experiments on the effect of SNP treatment of seedling of *Kosteletzkya virginica* significantly increased the K^+ content and decreasing Na^+ levels in both shoots and roots under salt stress (Guo et al, 2009).

The photosynthetic capacity was dependent on K^+ content, as observed in spinach leaf (Chow et al. 1990). CO_2 assimilation rate is also known to be dependent on K^+ homeostasis as its deficiency is associated with reduced CO_2 assimilation capacity reported in sugar beet (Terry and Ulrich 1973), cotton (Bednarz and Oosterhuis 1999) and barley (Degl'Innocenti et al. 2009) under salt stress. However, in the current investigation, no significant effect on CO_2 assimilation was observed.

The efficacy of NO as foliar spray may be jeopardized due to photo-instability and short life span of this reactive molecule. The biomass of the wheat plants treated with a foliar spray of NO increased under non-saline conditions as compared to saline conditions. This has suggested the participation of NO in the regulation of plant growth through its reaction with oxygen shortens its life span. The absence of the ameliorating effect of NO under non-saline

conditions in the current study points to the significance of dose dependency of NO in exhibiting its positive effect. The interplay between the protective and toxic effect of NO is dependent on its concentration and target site as observed in plants and animals (Wink et al. 1993; Beligni and Lamattina, 2001).

The correlation of photochemical efficiency (Fv/Fm) and relative chlorophyll content (SPAD) with tolerance response of plants to environmental stress has been well established regarding plant species and environmental stress factors. As recorded in literature and previous trials, exogenously applied NO is capable of increasing salt tolerance in plants (Manjunatha et al. 2008; Duan et al. 2007; Farooq et al. 2009). This is hypothesized to be a reflection of the ability of NO to conserve the efficiency of the photosynthetic drive of the plants under salt stress. Although a precise conclusion linking NO with photosynthesis is not possible at this stage, the nature of the analysis and the theory of varying evidence from the literature suggest that if experiments were carried out under more controlled conditions, a definite link between salt tolerance and NO influence on photosynthesis would be deciphered. This study was conducted to test this hypothesis.

The restricted symplastic/apoplastic uptake of ion (NO) has been considered for its failure on plants in the field. To ascertain if NO was ineffective in imparting salt tolerance in either of the species or it is due to challenges in its uptake/entry to tissue/cellular level, we carried out Petri dish experiments using excised leaf discs which invariably showed many promising results. The established mechanisms that can be contributing to this positive effect in leaf discs are reduced ROS accumulation by activating antioxidant enzymes. By quantifying the hydrogen peroxide and superoxide levels, employing fluorescent dyes the role of NO in tackling oxidative stress could be ascertained. The reason for the success of NO in excised leaf segments requires additional data generated by measuring the steady-state K^+ and Na^+ fluxes from the mesophyll of the leaf treated with NaCl and without SNP application.

Conclusion

The knowledge about the role of NO in plant physiology is validated by a large number of scientific publications. However, several knowledge gaps still persist in this area, and the present study was an initiative in this direction. Although the effect of nitric oxide on plants in a controlled environment is confirmed through publications, its efficacy in the field is

ambiguous, and so is the quantification and source of generation. The results of the present study indicate that application of SNP as a foliar spray on the intact plants of barley and pea had no significantly beneficial effects against NaCl toxicity and it was reflected in the growth, CO_2 assimilation, chlorophyll content and photochemical efficiency of these plants under salt stress. The exogenous application of NO is most likely challenged by its photo-instability and dose-time dependency factors. NO may indeed ameliorate salt stress effects in controlled environments as being proven in many publications, but its use as a spray to counteract salt stress in the field is still questionable.

Chapter 4: Cytokinin application increases salinity tolerance in pea by improving photosynthesis via increased K^+ retention in leaf mesophyll

Abstract

The current study was designed to investigate the effect of CYT mitigation of oxidative stress in pea mesophyll under salinity. CYT effect has been found to be dose dependent. Therefore, its concentration was optimized and further employed to check its impact on leaf ionic relations and whole-plant physiological characteristics. The application of 20 µM CYT had a positive impact on physiological features such as CO_2 assimilation, photochemical efficiency, and chlorophyll content ($P< 0.005$) as observed in glasshouse experiments. Laboratory studies using non-invasive MIFE ion flux measuring technique showed that Na^+ exclusion ability of pea mesophyll increased significantly ($P<0.05$) in CYT (20 µM) pre-treated plants as compared to that of non-treated, presumably by induction of SOS1 Na^+/H^+ exchanger. CYT treatment also had a beneficial effect on K^+ retention and maintenance of the cytosolic K^+ /Na^+ homeostasis in mesophyll cells. This retention originated from CYT-induced enhancement of H^+-ATPase activity, leading to an increase in the H^+ efflux and membrane re-polarization. Ultimately, this decreases the K^+ efflux through depolarization activated KOR channel on CYT treatment. The flux response of Ca^{2+}, H^+ & K^+ was further monitored to check whether CYT treatment can modulate the sensitivity of mesophyll to oxidative stress. It is shown that CYT provided a protective effect by minimizing the efflux of K^+ induced by H_2O_2, but not hydroxyl radicals. The implications of these findings are discussed.

Introduction

Plant hormones play a crucial role in adaptive responses of the plant (Kuiper et al., 1990) and cytokinins (CYT) represents one of the significant plant hormones contributing towards leaf senescence, stomatal opening, shoot morphogenesis, and cell division. CYT responds to the abiotic stress by evoking few signalling pathways (Ha et al., 2012; Hwang et al., 2012; Zwack and Rashotte, 2015). Many studies reported a decrease in endogenous CYT concentration under abiotic stress led to stress tolerant phenotype (Kudoyarova et al., 2007; Merewitz et al., 2011; Nishiyama et al., 2011). In contrast, a rise in the endogenous

levels of CYT and overexpression of biosynthesis genes - adenosine phosphate-isopentenyl transferases (IPTs) has been reported in plants under severe stress (Dobra et al., 2010). Kinetin, one of the types of CYT, is known to enhance the growth of crop plants under salinity significantly, soil waterlogging and soil acidity (Gadallah 1994; Salama and Awadalla 1987). Similarly, enhanced levels of zeatin have been reported in few Mediterranean shrubs under salinity stress (Lopez-Carbonel et al. 1996). The salt resistant barley with stunted growth under salt stress showed reduced levels of zeatin (Kuiper et al., 1990). Exogenous application of gibberellins and CYT aided plants to grow in NaCl rich soil.

Systemic responses are elicited by CYT by activating transcription of stress-inducible genes (Hare et al., 1997). Water deficit conditions in plants reduce the CYT levels, and overexpression of isopentenyl transferase (IPT) involved in CYT generation could enhance stress tolerance (Havlová et al., 2008; Albacete et al., 2008). Expression of IPT has to be under tight regulation to confer tolerance as higher concentrations than optimal decrease root growth and lead to water deficit. The expression of IPT driven by SAG12 (senescence promoter) and SARK (maturation promoter) resulted in the delay of photosynthetic decline due to age, thus reducing the yield loss of crop plants facing water deficit (Xu et al., 2009; Wingler et al., 1998)

The inhibition of leaf growth and premature senescence were linked to the fallen concentrations of CYT in leaf, root, and xylem sap under salinized conditions (Albacete et al., 2008). CYT is synthesized in roots and influences shoot responses such as leaf senescence stomatal movement and photosynthetic yield by increased levels in xylem sap under salinity. The CYT finds an easy way to reach transpiration stream as meristematic tissues present in root and cambial regions, which are the site of CYT biogenesis, are in close proximity to the xylem (Incoll and Jewer, 1987). The augmented synthesis and CYT transport provided a strategy to ameliorate the effects of salt stress on plant growth and yield. The enhanced expression of IPT gene escalates transport rate of CYT from root to shoot, which resulted in salt tolerance with increase in vegetative and fruit growth along with a delay in leaf senescence. The stomatal conductance is maintained as well as PSII efficiency, which delays the toxic ions accumulation (Albacete et al., 2008; Munns and Tester, 2008). Abscisic acid (ABA) is a phytohormone playing a significant role in various stress response signalling. ABA application results in cell desiccation and osmotic imbalance mimicking the abiotic (salinity, cold, drought) stress effects. This indicates the cross talk between the ABA and signalling factors evoked by

stressful conditions having same expression pattern (Shinozaki and Yamaguchi-Shinozaki, 2000).

The studies have indicated interplay between CYT and ABA in various signal transduction pathways. CYT act antagonistic to ABA in several physiological processes in plants; these also include the processes in response to stress. The elevated levels of CYT downregulated the ABA-responsive genes1 whereas a decrease in the same led to ABA hypersensitivity up-regulation of the stress responsive genes (Nishiyama, et al. 2011). The enhanced tolerance to the salt stress has been attributed to the hypersensitivity to ABA in CYT mutants. The profound effect of the stress amelioration in these mutants was achieved due to the ABA-dependent AREB activation (Tran, et al. 2010). In response to osmotic stress, the majority of ABRE-dependent gene expression are mediated by ABA. The drought stress increases ABA concentration, and the level of CYT decreases in defense to osmotic stress. However, whether the enhanced levels of the ABA play a role in the down-regulating CYT signaling, remains to be resolved. The positive type-B response regulators of CYT biosynthesis pathways have been observed to negatively influence the response to salt stress (Urao, et al. 1999). The escalation in the net CYT content down-regulates the ABA and stress responsive genes (ABI5) evoked by it. One of the signaling factor -histidine kinase 1 (AHK) of CYT signaling - has a positive influence on the regulation of drought and salt stresses (Kumar et al., 2013).

The oxidative stress is posed by salinity due to the generation of reactive oxygen species (ROS) such as hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), superoxide radical (O_2). Arabidopsis CYT-deficient mutant *ipt1,3,5,7* has hampered ROS scavenging mechanism indicating the cross- talk between ROS scavenging and CYT signaling (Nishiyama et al., 2012). Enhancement in the activities of antioxidative enzymes ascorbate peroxidase (APX) and catalase (CAT) in dark-induced senescence on exogenous applications of CYT has been reported (Zavaleta-Mancera et al., 2007). The same pattern of the activities was observed in both the antioxidative enzymes APX and CAT after heat stress (Liu and Huang, 2002). The upregulation of the CYT degrading genes CKX in transgenic Arabidopsis had a negative impact on the activities of antioxidants due to declined CYT levels (Mýtinová et al., 2010; Lubovská et al., 2014). These observations indicate the crucial role of CYT in ROS management in plants under environmental stresses.

Considering the crucial roles of CYT on multiple physiological and growth processes of plants, an attempt has been made to explore the impact of synthetic CYT on salinity and ROS- induced stress tolerance on plants. First, the influence of CYT in negating the impact of salt stress physiological aspects such as biomass yield, photosynthetic efficiency, and CO_2 assimilation in pea plant has been investigated at the whole-plant level. Then, the impact of CYT on leaf ionic relations was investigated at the tissue level by measuring the kinetics of H^+, Na^+ Ca^{2+} and K^+ transport across mesophyll cell plasma membranes in response to NaCl and ROS treatments.

Materials and methods

Plant materials and growth conditions

Pea (*Pisum sativum* L. cv. Onslow) seeds were obtained from the commercial supplier (Hollander Imports, Hobart, Australia). Seeds were surface-sterilized before seeds were sown at 20 mm depth in a 4 L pot containing potting mixture including 70% composted pine bark, 20% course sand, 10% sphagnum peat (pH 6.0) which was fertilised (1.8 kg m-3 dolomite, 6.0 kg m-3 Osmocote Plus and 0.5 kg m-3 ferrous sulphate) and thinned to 8 healthy seedlings. Plants were grown under controlled greenhouse conditions in the glasshouse at the School of Land and Food, University of Tasmania. Control plants were irrigated daily with tap water. Before treatments plants were grouped according to different treatments of salt and CYT. After two-leaf stage plants were irrigated daily with water containing various levels of salinity (NaCl) and a foliar spray of CYT (20 & 50 μM) solution was applied simultaneously for three weeks. To maintain the consistency foliar spray of distilled water was sprayed on control plants. A randomized complete block design was used, with four replicate pots for each treatment.

Measurements of physiological parameters

Mettler BB2440 Delta Range balances (Mettler-Toledo, Griefensee, Switzerland) were used to measure the fresh and dry weight of shoots. Shoots were dried at 65ºC for 2 days in Unitherm Dryer (Birmingham, UK). Young fully expanded leaves were used to extract sap as described by Cuin *et al.* 2009, in the Eppendorf tubes, and then centrifuged at 7000g for ten minutes. A vapour pressure osmometer (Vapro, Wescor Inc. Logan, Utah, USA) was used to measure cell sap osmolality. Flame photometer (PFP7, Jenway, FelstedDunmow, Essex,

England) was used for the quantification of leaf sap Na^+ and K^+ content. Stomatal conductance (Gs) was measured on the first fully expanded leaf using a Decagon leaf porometer (Decagon Devices Inc., WA, Australia) under constant light conditions (artificial light of 150 µmol m−2 s-1). LI-6400XT infrared gas analyser (Li-Cor Inc., Lincoln, NE, USA) was used to measure CO_2 assimilation (A), from first fully expanded leaf during the daytime (11 am – 2 pm). Relative chlorophyll content (SPAD) and maximum photochemical efficiency of photosystem II (PSII; chlorophyll fluorescence Fv/Fm ratio) were measured on a first fully expanded leaf by using a Minolta Chlorophyll Meter SPAD-502 (Minolta, http://www.konicaminolta.com/) and Chlorophyll Fluorometer (OS-30p, Opti-Sciences, USA), respectively.

Ion flux measurements

Abaxial epidermis was peeled off using forceps from the fully expanded leaf. 5x7 mm segments were quickly cut from peeled leaves and placed in 35mm petri dishes containing Basic Salt Media (BSM) solution (1 mM NaCl; 0.5 mMKCl; 0.1 mM$CaCl_2$; pH 5.7 non-buffered) on peeled side down floating in the dark overnight. 12 hours were sufficient for all confounding wounding responses to be stopped for measurements on MIFE. 5x7mm segments were mounted in a Perspex sample holder and placed in a 10-ml Perspex measuring chamber containing BSM solution. Treatments were applied by pipetting the required volume from stock solutions according to the experiments. Prepared and calibrated ion selective microelectrodes were placed in a line parallel to root axis with 40 µM distance between tips and root surface (mature zone) along with a maintained gap of 1-2µM between microelectrodes tips. Flux measurements during the first minute after treatment were discarded and presented as a gap in the figures.

Membrane potential measurements

Microelectrode with a tip of nearly 0.5µm was filled with 1 M KCL and mounted in MIFE. It was impaled into the mesophyll tissue by a manually-operated micromanipulator (MMT-5, Narishige, Tokyo, Japan). CHART software was used to monitor membrane potential while impalement and membrane potentials were measured for at least 30 seconds in every experiment to achieve steady readings.

Measuring net Na^+ efflux in 'recovery' experiments

As described in Cuin et al. (2011) and Shabala et al., (2007), the pea leaf abaxial epidermis was gently removed and 5×7 mm mesophyll segments were cut and left floating in a shallow Petri dish filled with BSM and 50 mM NaCl solution for overnight with and without CYT (20 µM). Mesophyll segment was rinsed three times in 10 mM $CaCl_2$ to remove surface NaCl. The mesophyll segment was then transferred to a clean Perspex chamber containing Na^+ free BSM solution in 2 minutes for MIFE measurements.

Statistical Analysis

Data were statistically analysed using analysis of variance (ANOVA), and significance difference was compared at 5 % probability level using Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) software. Graphs were made using Microsoft Excel, v 2010, and error bars in the graph represented standard error of four replications per treatment. Means sharing a common letter in the column were not significantly different at 5% probability. Error bars represented S.E.

Results

Different concentrations of CYT have been tested on pea plants subjected to various levels of salinity stress. Biomass yield, CO_2 assimilation, photochemical efficiency, and ion fluxes have been measured for evaluating the CYT effect. The influence of CYT in mitigating the ROS-induced stress on pea generated by H_2O_2 & hydroxyl radicals generated by Cu/A (0.3/1 mM) mix was also investigated.

Figure 1.1-: Effect on pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until images were taken. Means ± SE (*n* =6).

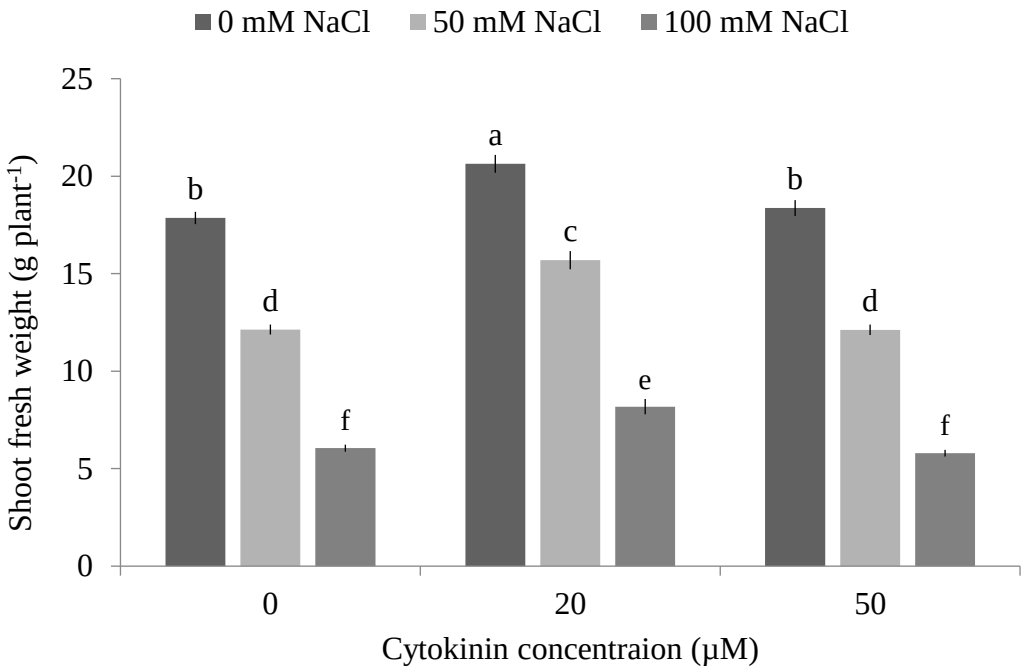

Figure 1.2-: Effect on fresh weight of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at $P < 0.05$

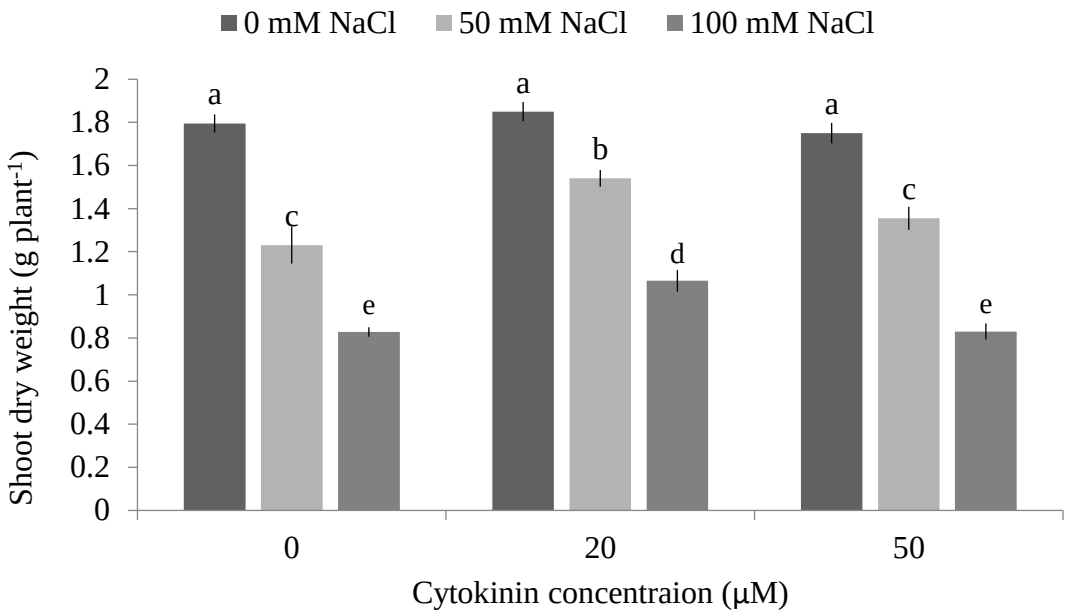

Figure 1.3-: Effect on dry weight of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at $P < 0.05$

Pea biomass production in response to CYT application under salt stress

Three weeks of treatment with different concentrations of NaCl (0, 50 and 100 mM) has induced a considerable reduction in fresh and dry weights of pea plants ($P < 0.05$) (Fig. 1.2& 1.3). Furthermore, pea biomass was reduced by 25 % and 65 % when exposed to 50mM NaCl and 100 mM NaCl stress level respectively. Nonetheless, CYT application significantly ameliorated negative effects of salt stress in pea plants and increased pea biomass production by improving plant fresh and dry weight (Fig. 1.2 & 1.3). Among CYT application levels, application of 20 µM CYT substantially increased fresh shoot and dry weight under salt stress, while application CYT concentration of 50 µM showed similar results regarding pea biomass, as was observed without the application of CYT under salt stress.

Ameliorating effect of CYT on chlorophyll content, photochemical efficiency, and CO_2 assimilation.

Photochemical efficiency of PSII (chlorophyll fluorescence Fv/Fm ratio) represents one of the vital parameters monitored for assessing the physiological status of the plants. A

significant reduction (25% at 50mM NaCl and 50% at 100mM NaCl) in the Fv/Fm ratio of the pea leaves has been recorded in the control samples. However, treatment of 20µM spray of CYT has helped to recover the ratio by approximately 12.5% (Fig 2.1), thereby preventing detrimental effects on leaf photochemistry. However, photochemical efficiency could not be recovered to the safe level in pea plants exposed to severe salt stress (100 mM) by CYT application. Interestingly, treatment with 50µM of CYT did not result in proportional enhancement of the Fv/Fm ratio. Therefore, it can be concluded that the efficacy of CYT is dose-dependent and efficient at 20µM.

In comparison to the control plants, chlorophyll content of the pea plants exposed to salt stress (50 mM and 100 mM NaCl) was found significantly lower ($P < 0.05$) as illustrated in Fig 2.2. CYT treatment on the plants exposed to salinity increased the chlorophyll content, but could not reverse the effect of salinity as the values of chlorophyll content after CYT treatment were still lower than that of the control plants.

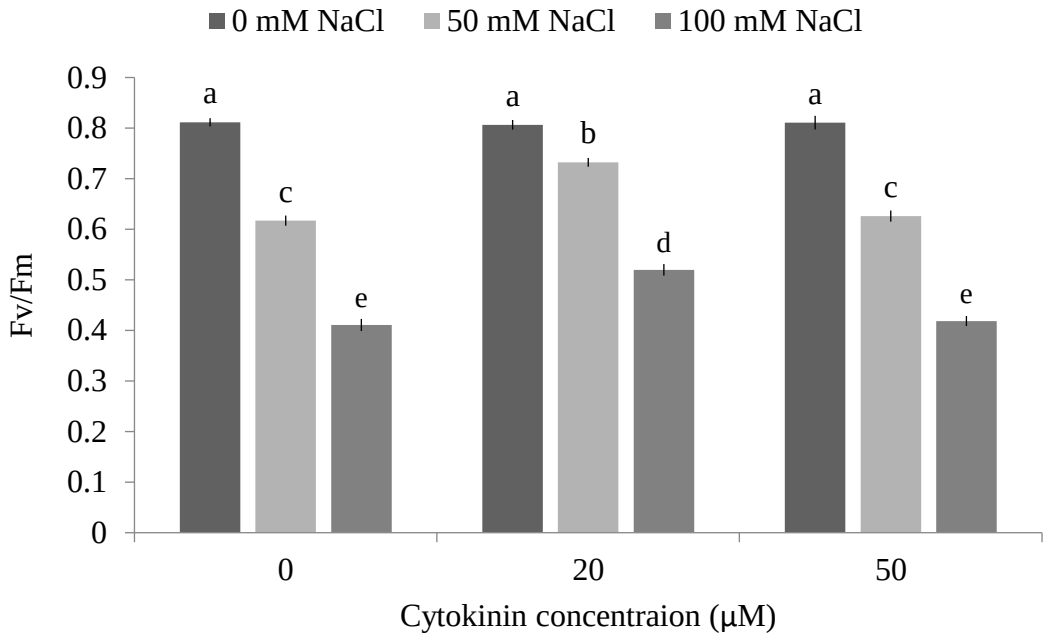

Figure 2.1-: Effect on the photochemical efficiency Fv/Fm of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (n = 6). Data with the different low case letters is statistically different at $P < 0.05$

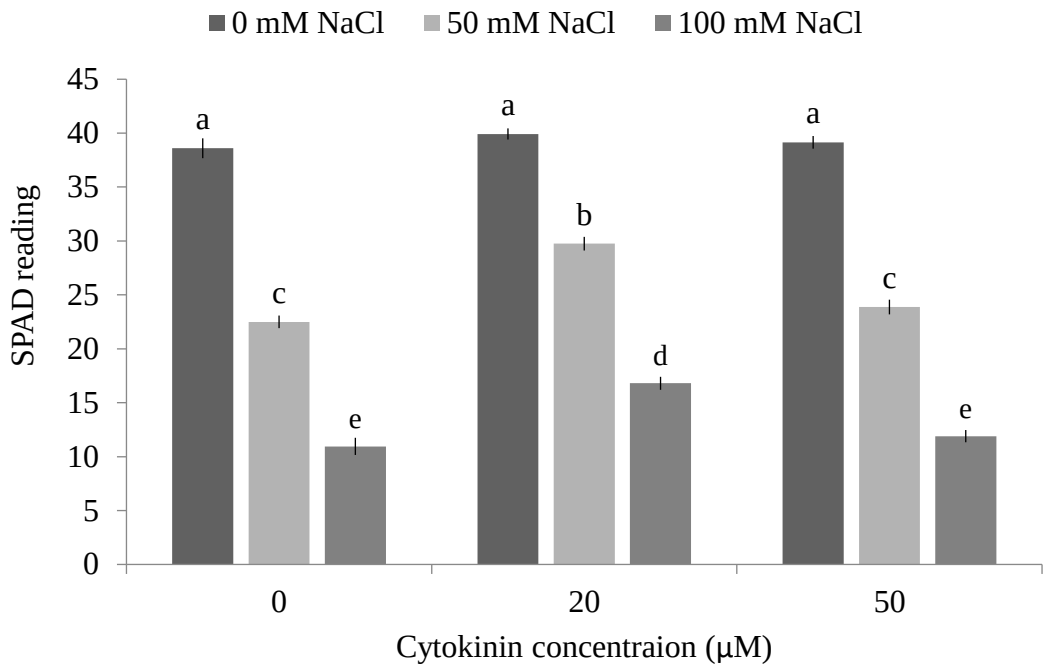

Figure 2.2-: Effect on the chlorophyll content SPAD of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at $P < 0.05$

The stomatal closure mainly causes the reduction in the CO_2 assimilation under water deficit. The plant response to CO_2 assimilation is considered similar to stomatal conductance under stress. The ameliorative effect of 20 µM foliar spray of CYT on CO_2 assimilation rate of pea plant (Fig 3) under 50 and 100 mM salt stress is evident due to its influence on the activity of RUBISCO and stomatal conductance.

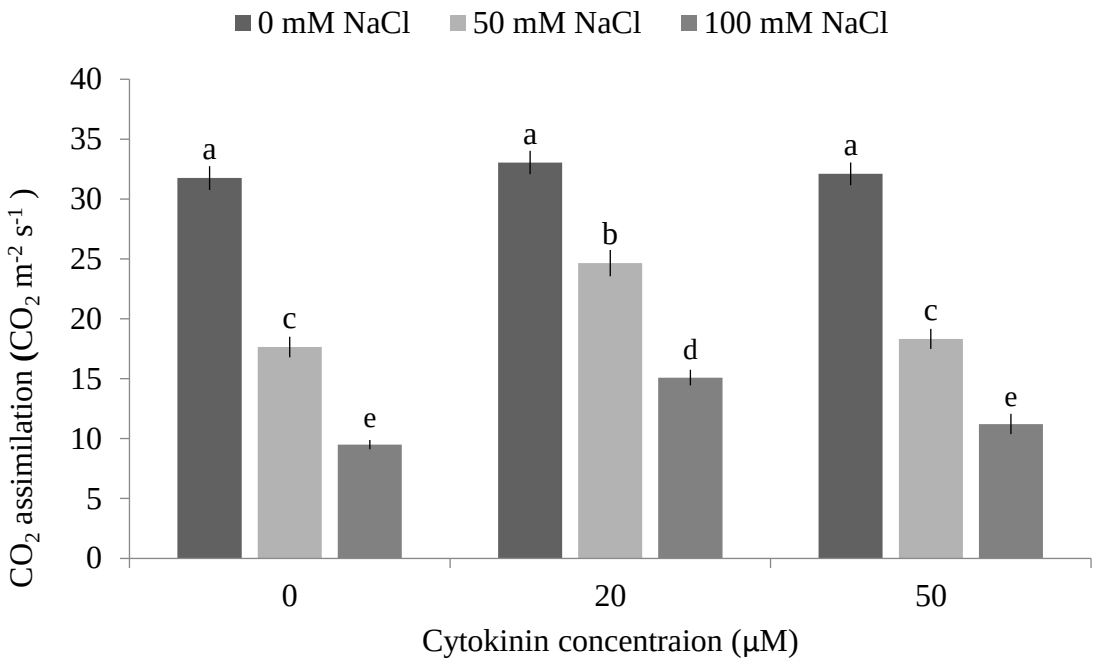

Figure 3-: Effect on CO_2 assimilation of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at $P < 0.05$

After four weeks of moderate salinity stress posed by 50 mM and 100 mM NaCl, an increase in the concentrations of pea leaf Na^+ was observed. Simultaneously, a drop in the K^+ levels was observed (Fig 4.1 & 4.2). The positive effect of foliar application of CYT on plants inflicted with salinity stress has been partly attributed to the regulation of K^+ transport (Green & Muir 1979) as observed in the present study.

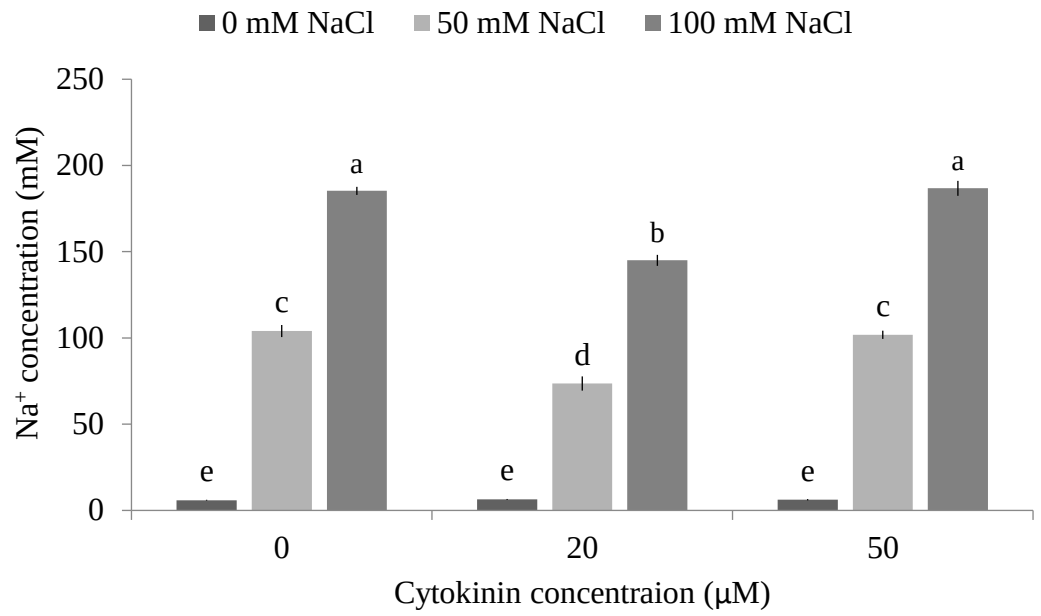

Figure 4.1-: Effect on Na^+ leaf concentration of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at P < 0.05

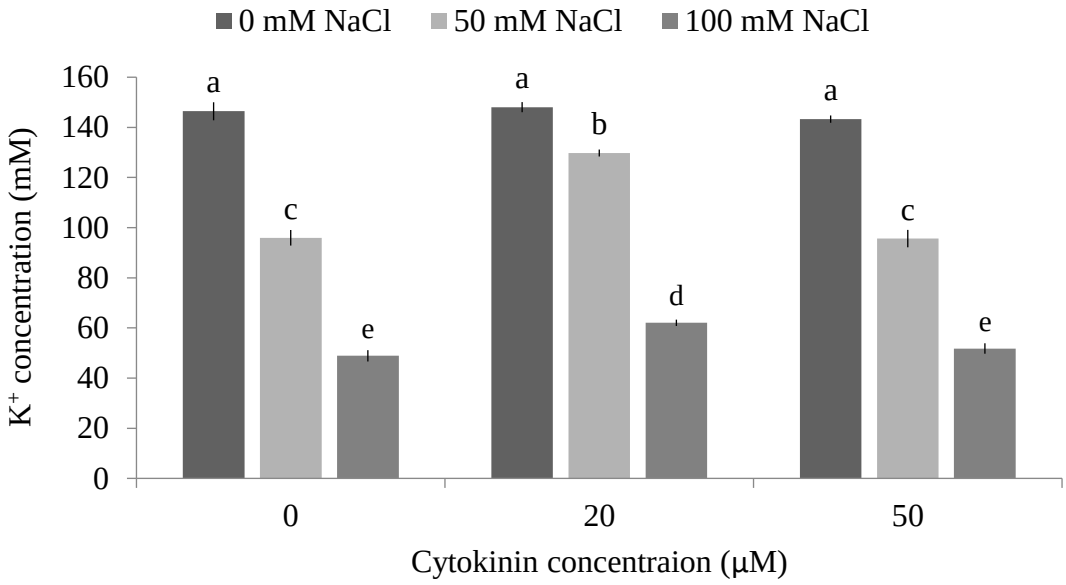

Figure 4.2-: Effect on K^+ leaf concentration of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at P < 0.05

Due to the uptake of the Na^+, the osmolality of leaf increases under saline conditions (Fig 5). The foliar application of 20 µM CYT has been observed to limit the uptake of Na^+ to the mesophyll cells of pea plant subjected to salinity probably by regulating the ion channels present in the plasma membrane. Unlike the outcomes of previous parameters assessed, leaf osmolality did not show any notable difference among different doses of cytokinin, with respect to 100 mM NaCl stress.

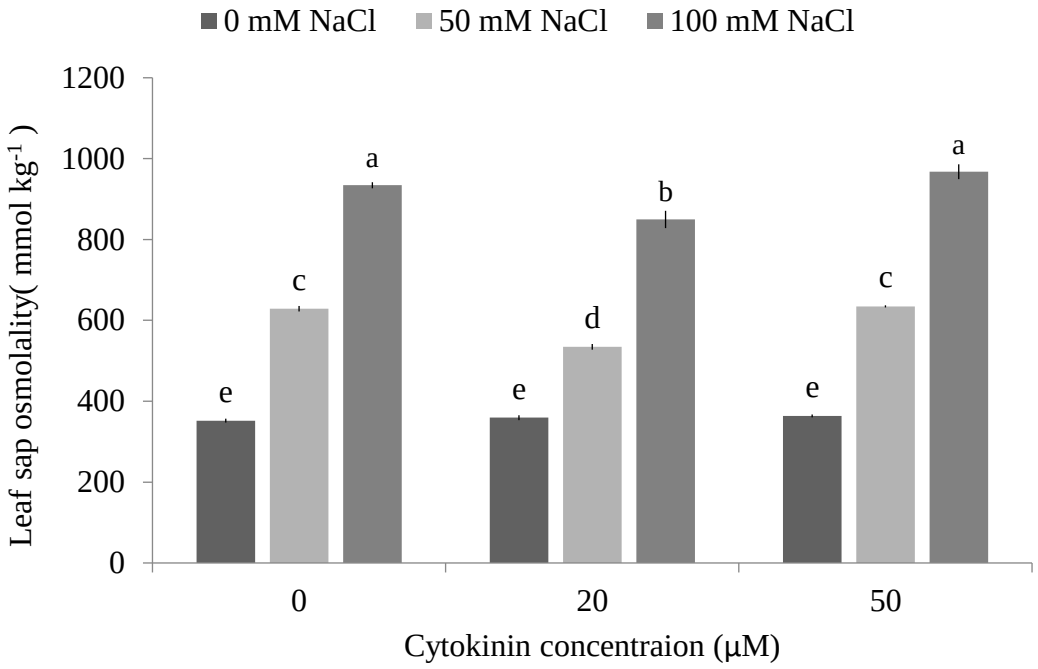

Figure 5-: Effect on leaf sap osmolality of pea (cv Onslow) plants grown for four weeks in glass house under various levels of salinity (NaCl) & CYT foliar spray treatments (three weeks) until measurements were taken. Means ± SE (n = 6). Data with the different low case letters is statistically different at $P < 0.05$

Salinity and CYT induced kinetics of Na^+, K^+, H^+ and Ca^{2+} fluxes

To investigate the role of CYT in the regulation of Na^+ transport from apoplast into mesophyll, Na^+ flux response was measured from pea mesophyll using the MIFE technique. The comparison was made after 12-hrs of overnight treatment in Na^+ free BSM solution with and without CYT (20 µM) in response to (50mM) NaCl stress. The pre-treatment of 50 mM NaCl before application of CYT (20 µM) to pea mesophyll led to an influx of Na^+. The application of CYT could not control the influx of Na^+ on NaCl treatment. The analysis of Na^+ flux response is misleading due to high flux noise for Na^+ due to its presence in the solution (Fig 6.1). NaCl stress (50 mM) on pea mesophyll resulted in large K^+ efflux, but 12 h pre-treatment of pea mesophyll with CYT reduced the NaCl induced K^+ efflux (Fig. 6.2). No clear trends have emerged on H^+ and Ca^{2+} efflux upon NaCl stress (50 mM) on pea mesophyll between control and CYT pre-treated pea mesophyll (Fig. 6.3 & 6.4)

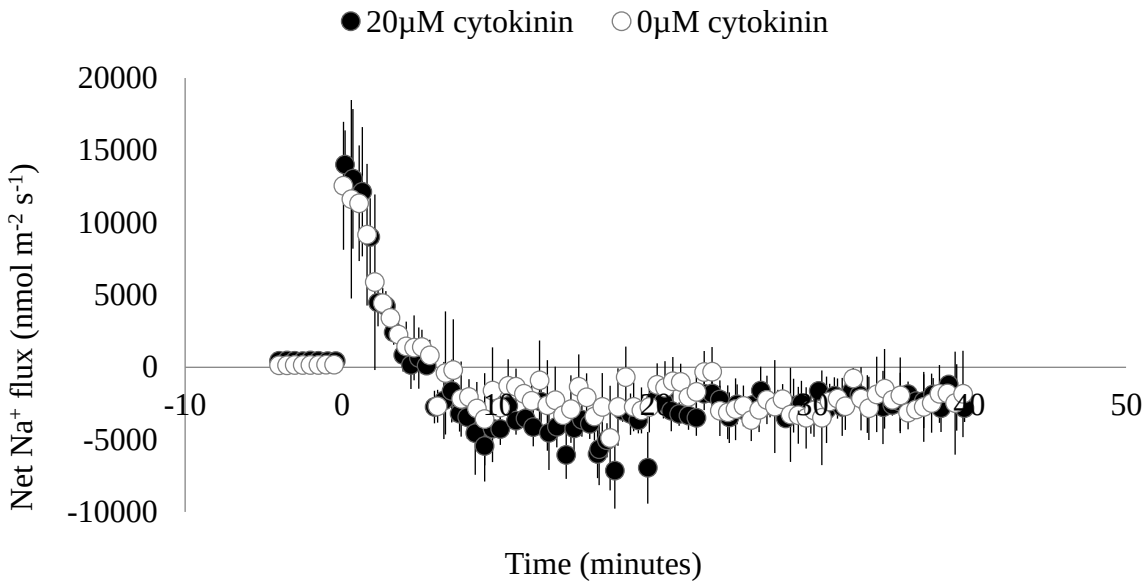

Figure 6.1 -: Net Na^+ fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to 50mM NaCl treatment. The sign convention is efflux negative. Means ± SE (*n* = 6).

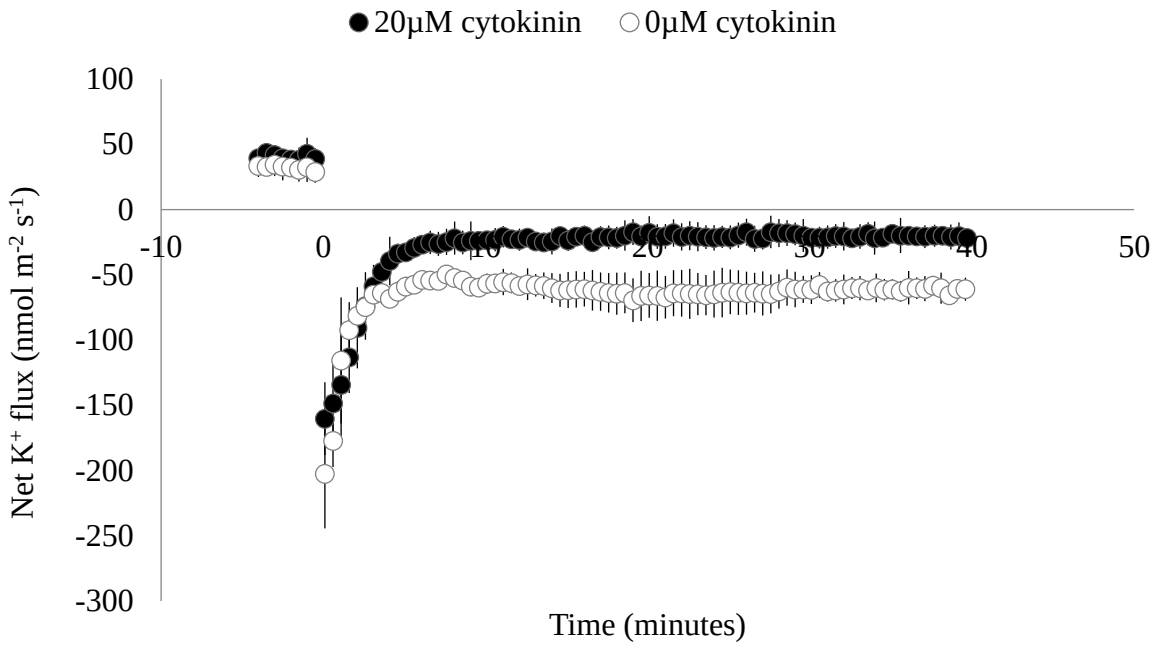

Figure 6.2 -: Net K^+ fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to 50mM NaCl treatment. The sign convention is efflux negative. Means ± SE (*n* = 6).

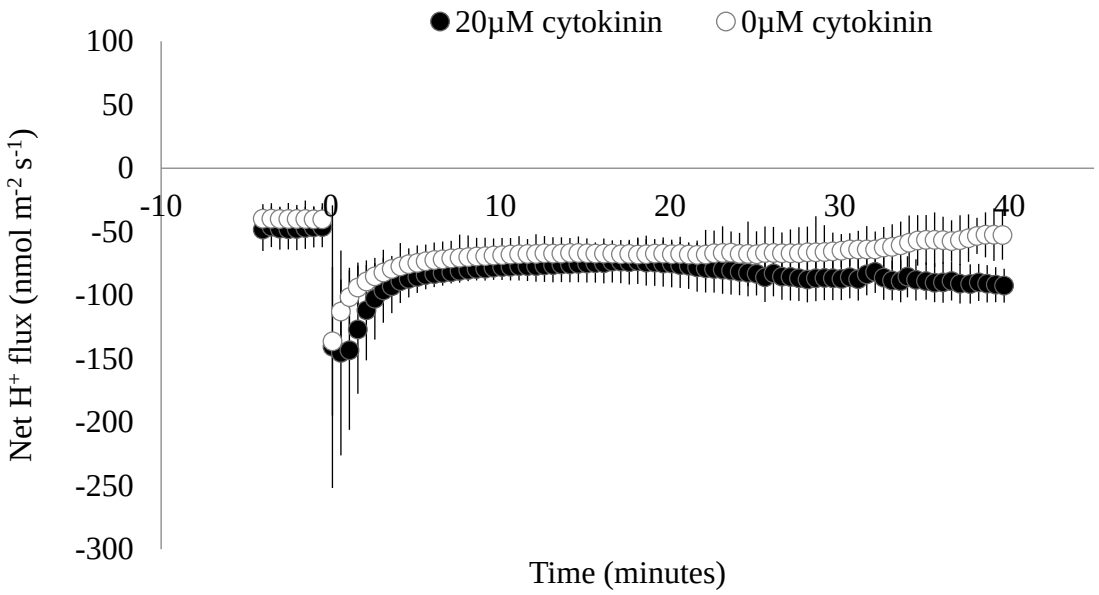

Figure 6.3 -: Net H^+ fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to 50mM NaCl treatment. The sign convention is efflux negative. Means ± SE (*n* = 6).

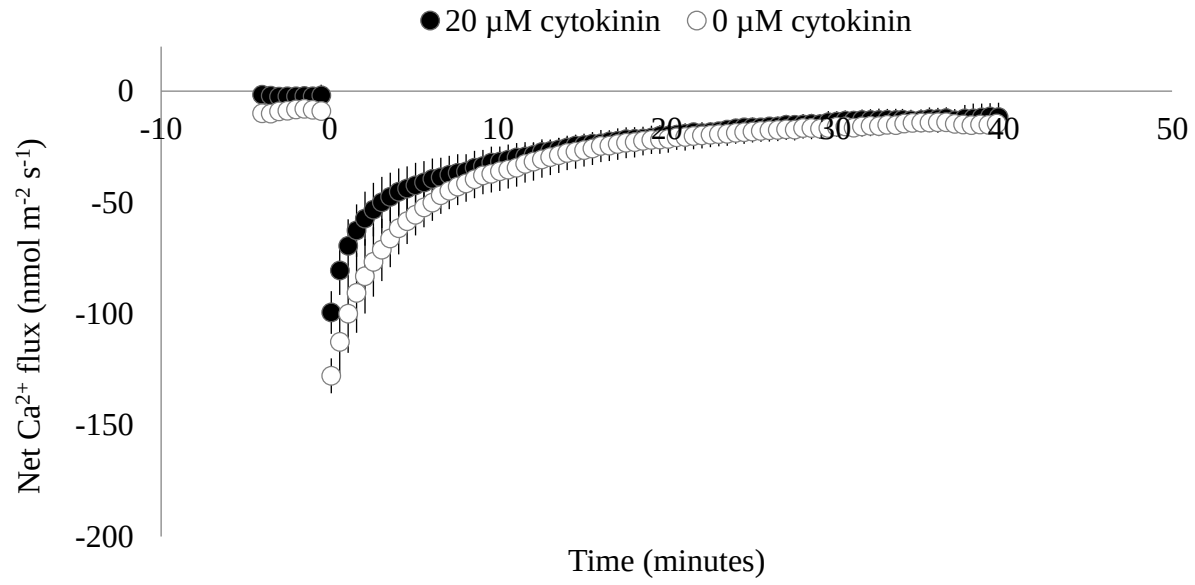

Figure 6.4 -: Net Ca^{2+} fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to 50mM NaCl treatment. The sign convention is efflux negative. Means ± SE (n = 6).

Effect of Cytoknin on K^+/Na^+ ratio in pea mesophyll

The recovery protocol estimated efflux of Na^+ by Na^+/H^+ antiporter SOS1 present in the plasma membrane of the pea mesophyll. In recovery protocol (Fig.7), as described in Shabala et al., (2007), Na^+ exclusion ability of pea mesophyll increased significantly($P<0.05$) in CYT (20 µM) pre-treated samples compared with that of non-treated. This indicated possible elicitation of SOS1 activity in pea mesophyll by CYT leading to the extrusion of Na^+ from the cytosol (Shabala, 2000).

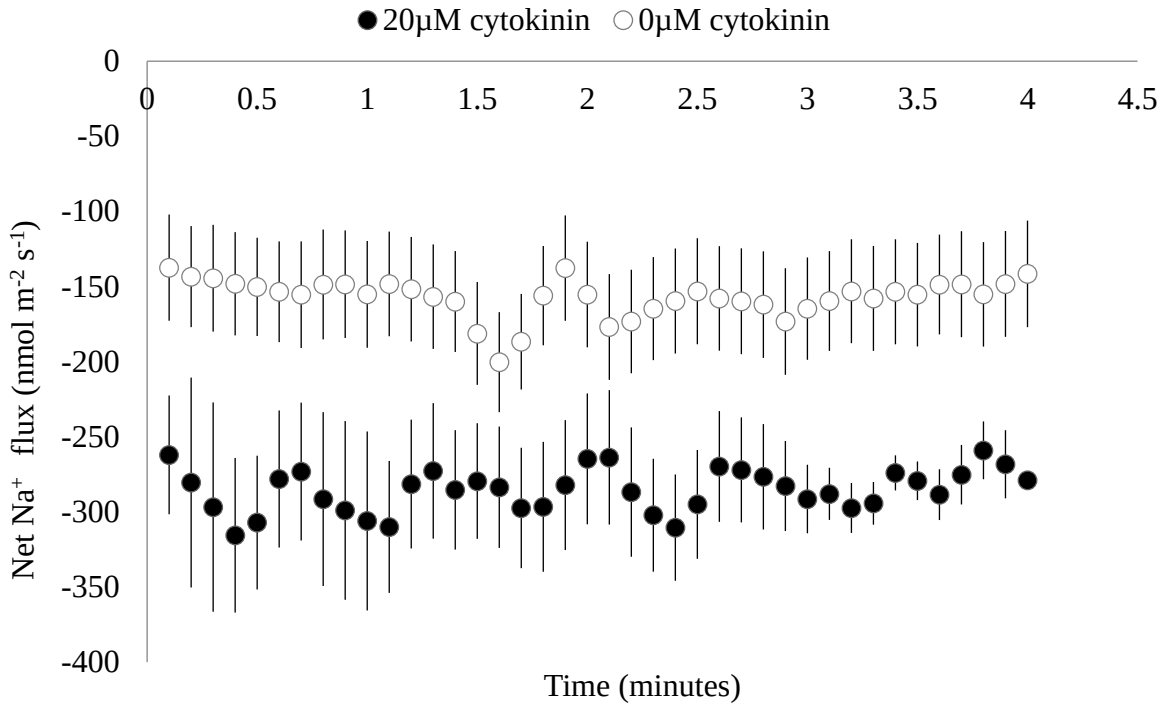

Figure 7-: Estimation of SOS1-like activity in pea mesophyll in so-called "recovery experiemnts". Net Na^+ fluxes were measured from mesophyll segments of pea (cv Onslow) leaf after receiving 12 h of cytokinin (0 & 20 µM) and 50 mM NaCl treatment Before measurements mesophyll segments were washed in $CaCl_2$ and transferred in to Na^+ free BSM solution. Means ± SE (n = 6).

Combined effect of NaCl (50 mM) stress pre-treatment (12 h) and CYT (20 µM) pre-treatment (12 h) on membrane potential kinetics of pea mesophyll was tested (Fig. 8). Pre-treatment of pea mesophyll with CYT (10 µM) increased the resting potential towards more negative value in comparison to the control.

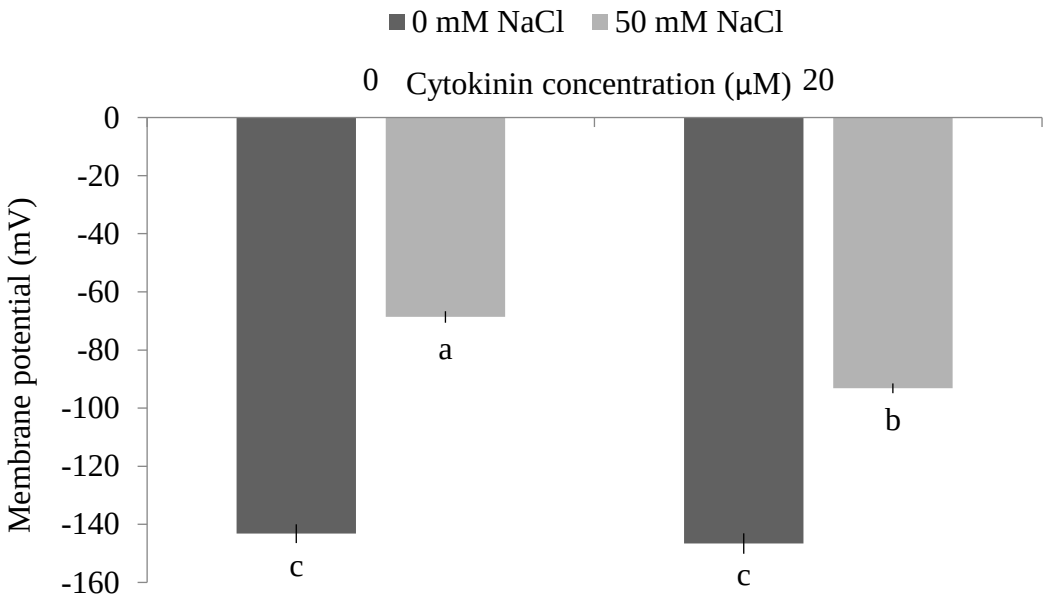

Figure 8-: Membrane potential measured from mesophyll segments of pea (cv Onslow) leaf after receiving 12 h of cytokinin (0 & 20 µM) and 50 mM NaCl treatment. Before measurements mesophyll segments were washed in $CaCl_2$ and transferred in to Na^+ free BSM solution. Means ± SE (n = 6).

Effect of CYT in mitigation of oxidative stress and maintenance of K^+ homeostasis

The MIFE technique was also employed to assess the effect of CYT on oxidative stress and K^+ homeostasis. Exogenous application of ROS (H_2O_2 (10 mM)) on pea mesophyll induced a significant K^+ efflux (Fig. 9.1). Pre-treatment of pea mesophyll with CYT (20 µM) showed lower efflux ($P<0.05$) in comparison to the control when H_2O_2 (10 mM) was applied. H_2O_2 induced K^+ efflux was not prompt like NaCl treatment as it developed steadily, reaching top values after 10 minutes.

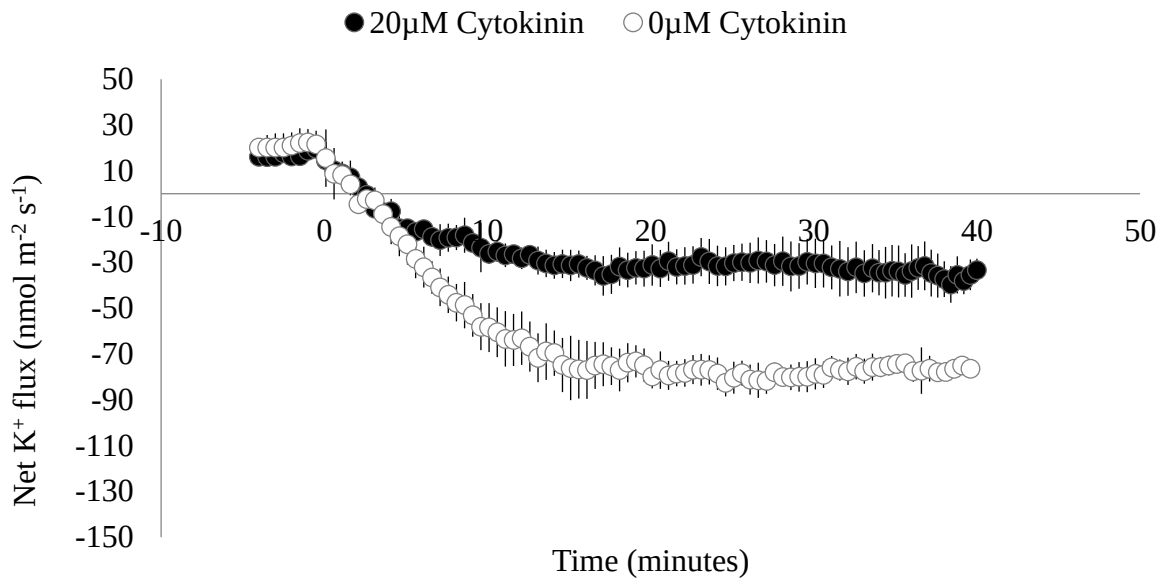

Figure 9.1 -: Net K^+ fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to H_2O_2 treatment. The sign convention is efflux negative. Means ± SE (*n* = 6).

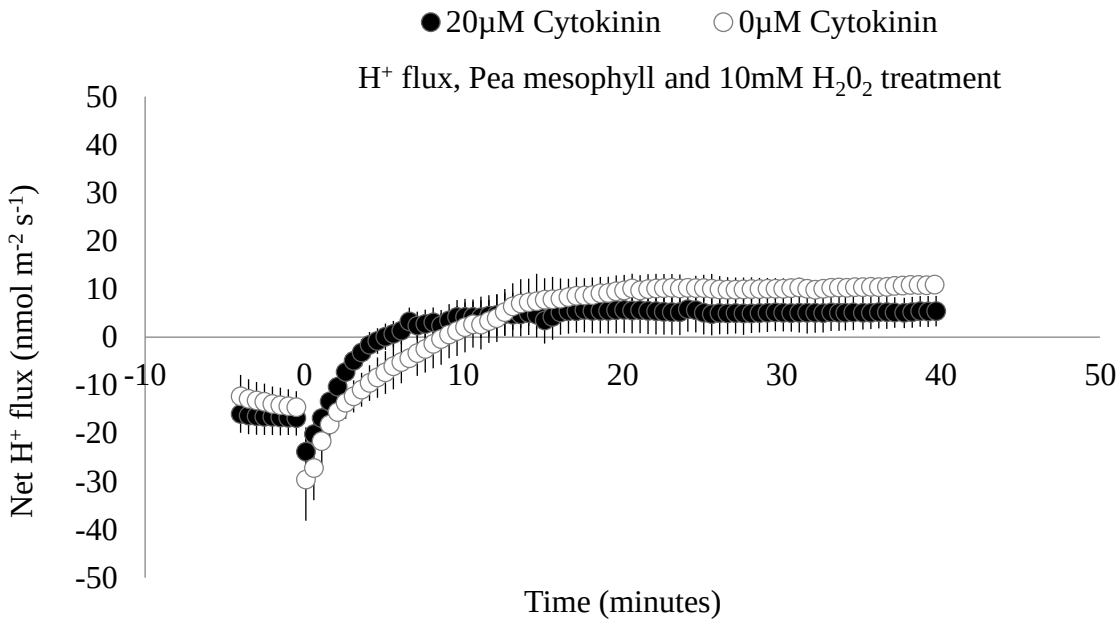

Figure 9.2 -: Net H^+ fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to H_2O_2 treatment. The sign convention is efflux negative. Means ± SE (*n* = 6).

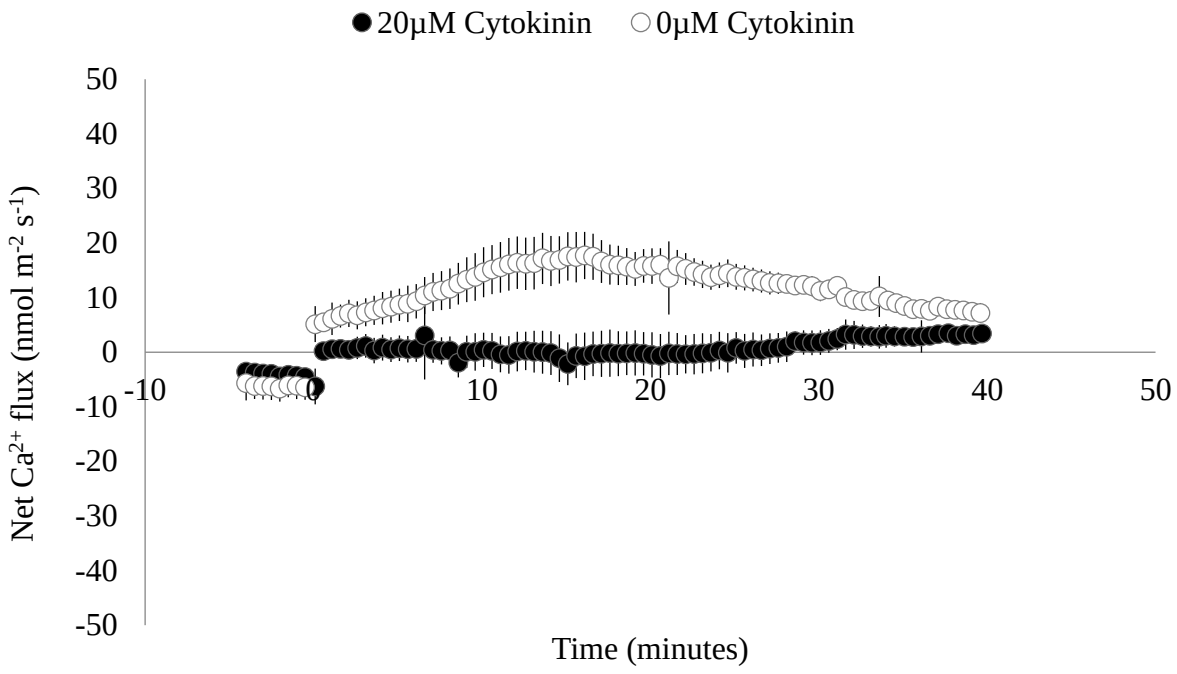

Figure 9.3 -: Net Ca^{2+} fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution in response to H_2O_2 treatment. The sign convention is efflux negative. Means ± SE (*n* = 6).

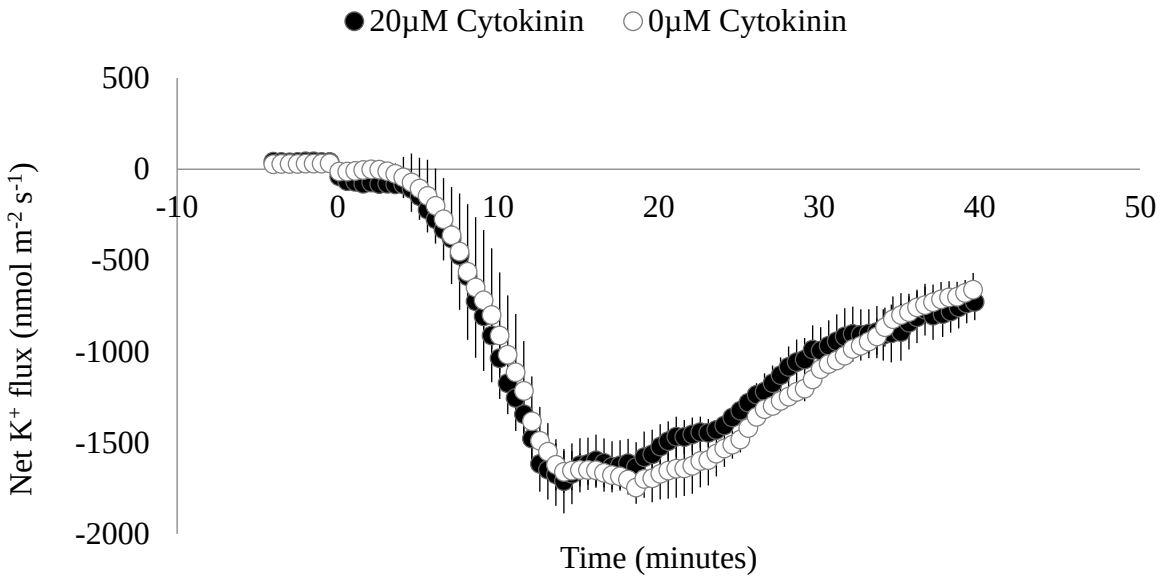

Figure 10.1 -: Hydroxyl radical-induced net K^+ fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^+ free BSM solution. The sign convention is efflux negative. Hydroxyl radical-generating Cu/A (0.3/1 mM) mix was added at time zero. Means ± SE (*n* = 6).

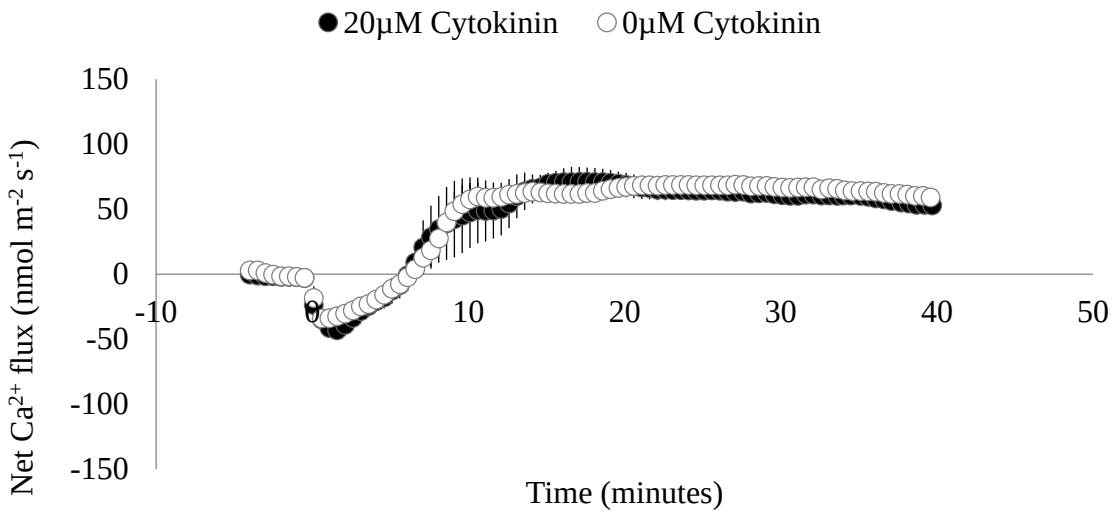

Figure 10.2-: Hydroxyl radical-induced net Ca^{2+} fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^{+} free BSM solution. The sign convention is efflux negative. Hydroxyl radical-generating Cu/A (0.3/1 mM) mix was added at time zero. Means ± SE (*n* = 6).

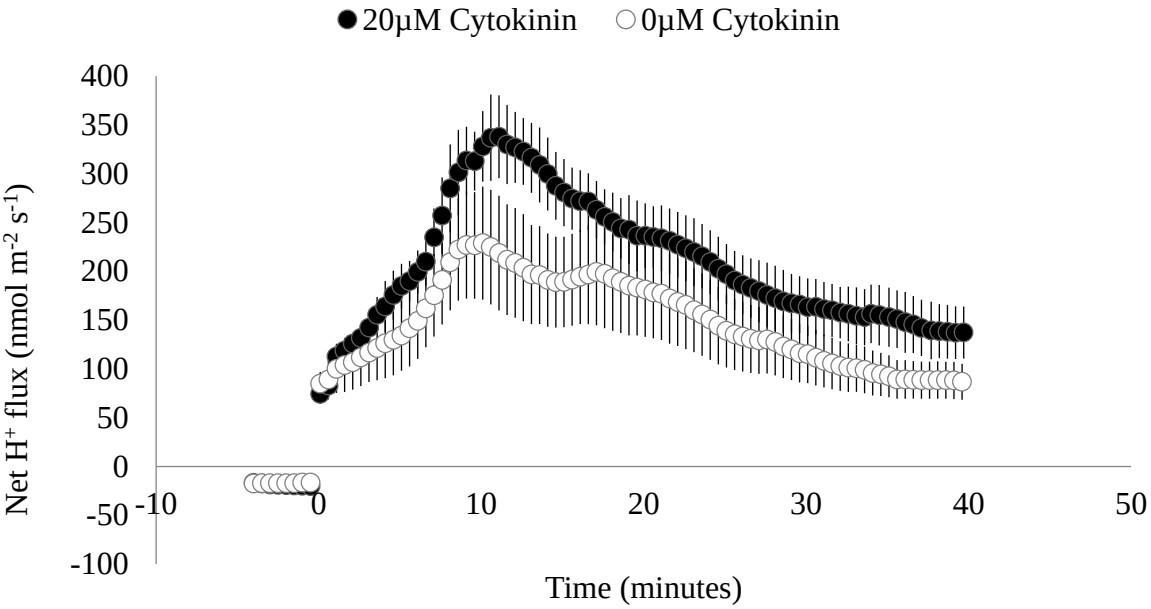

Figure 10.3 -: Hydroxyl radical-induced net H^{+} fluxes measured from mesophyll segments of pea (cv Onslow) plants after 12 hours cytokinin (0 & 20 µM) treatment in Na^{+} free BSM solution. The sign convention is efflux negative. Hydroxyl radical-generating Cu/A (0.3/1 mM) mix was added at time zero. Means ± SE (*n* = 6).

The hydroxyl radicals generated from the application of Cu/A (0.3/1 mM) led to massive K^+ efflux from pea mesophyll cells (Fig. 10 .1). The efflux of K^+ due to Cu/A (0.3/1 mM) treatment was not instant, but it reached peak values 15 min after treatment. However, there was no significant difference observed between K^+ net efflux of CYT & non-CYT treated samples. In the current ion flux measurement experiments, it has been observed that initially, when mesophyll cells of pea were introduced to oxidative stress, the influx of Ca^{2+} resulted in an increase in cytosolic Ca^{2+}. But as the time impends, efflux of the Ca^{2+} set in due to the disturbed membrane stability and it couldn't be managed with the application of CYT (Fig 10.2). The application of 10 mM H_2O_2 resulted in the regulated efflux of Ca^{2+} in CYT (20 µM) treated pea mesophyll cells as shown in Fig 9.2. CYT treatment enhanced the H^+ efflux rate, which is one of the significant adaptive mechanisms of salt tolerance in plants on Cu/A (0.3/1 mM) treatment (Fig 10.3), but no significant effect was observed with the H_2O_2 application in pea mesophyll cells (Fig 9.3).

Discussion

Phytohormones have been reported to contribute towards stress response mechanisms and adaptation in plants (Sharma et al., 2005; Shaterian et al., 2005). In an extensive review of the interactions of phytohormones and salinity stress among crops, Javid et al. (2011) have traced close inverse correlation between concentrations of auxin, CYT, gibberellins, and SA in the plants challenged with salinity stress and a direct association in the case of ABA and JA. CYT has been proved to play complex roles in abiotic stress tolerance mechanisms of plants and few cases of contradictory results as well (Zwack and Rashotte 2015). The present investigation is an attempt to decipher the salinity tolerance mechanisms conferred by CYT in pea at the plant level and on excised mesophyll tissue. The effect of CYT in the management of NaCl induced salinity was assessed regarding biomass yield, photochemical efficiency, O_2 assimilation, ions homeostasis, and countering oxidative stress. Biomass yield regarding the fresh and dry weight of the pea plants exposed to long salinity stress (3 weeks) during early development was significantly reduced, and a decrease was inversely proportional to the salt concentration applied. This outcome shares similarity with that of Arabidopsis exposed to salinity for 2 weeks (Bose et al. 2013). The imbalance created in the ionic relations within the plant tissues has been attributed to the growth and biomass reduction under salinity stress (Shabala et al. 2010). Similarly, reduction in the fresh/dry weight of the plants under salinity has been reported by earlier studies as well (Ali-Dinar et al. 1999; Chartzoulakis and

Klapaki 2000). Our experiments further compared the biomass of the pea seedlings under medium (50 mM) and high (100 mM) NaCl concentration and treatment of CYT with varying concentrations. The extent of reduction in the fresh and dry weights of the plants recorded significant difference under the influence of CYT (20 μM& 50 μM). Interestingly, 20 μM CYT gave better results with a lesser extent of biomass reduction in comparison with the control treatment. A similar influence of salicylic acid (SA) on biomass of Arabidopsis has been reported by Jayakannan et al. (2013). Therefore, it is apparent that both CYT and SA provide a positive impact in salinity stress management among plants and the effect is dose dependent. Consistently Salama and Awadalla (1987) have reported a significant increase in plant growth under salinity through the application of kinetin.

Na^+ exclusion from transpiration stream is the primary response of plants to salinity and can impart salt tolerance to a limited extent (Gorham et al., 1990; Shi et al., 2002). However, long-term salinity exposure elicits stress responses such as a substantial leak of K^+ from the cytosol (Shabala, 2000), inhibition of photosynthesis (Shabala et al., 2010), enhanced ROS production (Miller et al. 2010), and reduction in stomatal aperture (Brugnoli and Lauteri 1991). Leaf ion concentration estimates have indicated a reduction in Na^+ and a significant enhancement in the concentration of K^+ with 20 μM CYT application. This finding suggests the prevention of accumulation of Na^+ synergistic with retention of K^+ under the influence of the applied dose of CYT. A significant difference (Fig. 8) in efflux pattern of Na^+ under salt stress with and without CYT indicates CYT effect on modulating the SOS1 activity under salt stress and improved K^+ retention ability under salt stress, but minimal effect on H^+ pumping ability.

The fall in the photosynthetic rate under salt stress-induced water deficiency has been attributed to the stomatal limitation and impairment of RuBP and ATP synthesis (Lawlor, 2002). Exogenous application of CYT has been observed to counteract the water stress caused by salinity and alleviating its effect on the activity of the RUBISCO or PEP carboxylase, maintaining photo-efficiency of PS I and II as well as chlorophyll content (Metwally et al. 1997; Pandey et al. 2000). Salinity appears to interfere with photosynthesis via stomatal closure and diminution in the photochemical reactions and carbon assimilation (Megdiche et al., 2008; Stoeva and Kaymakanova, 2008). The chlorophyll content of the pea plants showed

similar trends as those of Fv/Fm ratio under combined treatment with two doses each of NaCl and CYT. In this case, the effective concentration of CYT was found to be 20 μM, ; at this concentration it could restore the chlorophyll content to a considerable extent. The salt-induced stress can break down chlorophyll (Chl), primarily due to the increased level of Na^+ (Yang et al. 2011), which turns toxic to the plants at higher concentrations. Earlier studies have reported a reduction in Chl a & b under salt stress in different crop plants such as sunflower (Akram and Ashraf 2011), alfalfa (Winicov and Seemann 1990), and wheat (Arfan et al. 2007, Perveen et al. 2010). Such reduction in chlorophyll content under salinity stress could be due to altered biosynthesis or accelerated degradation of the pigment (Ashraf and Harris 2013). The CO_2 assimilation among the salt-treated and controlled plants also showed a similar extent of differences to those of chlorophyll content in our current investigation. This may be directly linked with the reduction in chlorophyll content recorded under similar salt concentrations.

The presence of Na^+ in the cytosol disturbed the ion homeostasis and led to efflux of K^+ from mesophyll (Shabala 2000), with consequences for cell viability (Bose et al. 2014; Shabala and Pottosin 2014). Salinity leads to a decrease in K^+ content (Wu et al. 2013) and increase ionic strength within the cell, causing denaturation of proteins (Kronzucker et al. 2013). The influx of Na^+ through nonselective cationic channels (NSCC) under saline conditions causes a significant membrane depolarization leading to massive K^+ efflux from the cell through KOR channels (Shabala et al., 2006). The survival of plants under abiotic stresses strongly depends on their ability to activate the H^+-ATPase pump, to restore (otherwise depolarised) membrane potential and also provide a driving force for SOS1 Na^+/H^+ exchanger. The presence of Na^+/H^+ antiporter in the plasma membrane of plants is critical for their growth under high salinity as it removes toxic Na^+ from the cytoplasm. Salt stress increases the activities of plasma membrane H^+-ATPase and Na^+/H^+ antiporter (Zhang et al. 2006). Salinity also stimulates elevation in cytosolic Ca^{2+}, which results in the sudden increase in ROS levels (Tracey et al., 2008) from NADPH oxidase positive feedback mechanism leading to enhanced K^+ efflux through NSCC (Demidchik et al., 2003). The net decline in the cytosolic K^+ concentration activates caspase-like proteases executing PCD. NaCl induced PCD should be adequately attenuated in plants lacking functional KOR channels (Arabidopsis *gork* mutants) or by efficient scavenging of ROS carried out by antioxidant enzymes. The plants that maintain higher H^+-ATPase activity and have a negative membrane potential in salt stress were found to be more resistant to NaCl motivated PCD.

Therefore, the mentioned ions flux response is also measured under salinity and ameliorating effect of CYT in tackling ionic toxicity. The membrane potential is restored with the increase in H^+ -ATPase activity closing GORK-like channels (K^+ outward rectifying channels) (Shabala and Cuin 2008). It has been observed that H^+- ATPase enhanced activity down-regulate the GORK channels and simultaneously provides the driving force for Na^+/H^+ exchanger (SOS) at plasma membrane removing excess of Na^+ in the cytoplasm (Chen et al. 2007). CYT pre-treatment showed a positive impact towards the restoration of membrane potential in the pea mesophyll cells induced by salt stress. However, complete restoration could not be attained in the current experimental trial. Further optimization experiments are needed for the purpose.

Under salinity, the K^+ uptake by the plants is impaired leading to the oxidative stress and eventually programmed cell death. Cu/A generates $OH^{\bullet}$ (Halliwell&Gutteridge 1999), which is the most potent reactive oxygen species (ROS) generated in most of the plant species under stress (Shen et al., 1997; Van Doorslaeder et al. 1999). In the previous reports, Cu/A has been stated the cause of membrane depolarization leading to activation of K^+ outward rectifying channels and efflux of K^+ under oxidative stress (Cuin and Shabala, 2007). It also seems plausible that CYT could reduce Na^+ influx (Fig. 7) through NSCC by acting as ROS scavenger, thus reducing the membrane depolarization (Fig. 8) and K^+ efflux through KOR. CYT affect membrane potential by improving the Na^+ exclusion (mostly SOS1 mediated) from the cell, which is one of the major cause of membrane depolarization in the salt affected tissue. As SOS1 is highly salt-induced protein hence it is quite unlikely to have CYT effect under control condition therefore no significant effect was observed in control plants membrane potential.

CYT provides a protective effect by minimizing the efflux of K^+ induced by ROS (H_2O_2) induced stress in the pea mesophyll. Unlike the influence on K^+ flux response, CYT promotes efflux of H^+ induced by Cu/A (0.3/1 mM) in pea mesophyll cells. The inconsistency in the results obtained requires further repetition along with further experimentation to define the role of CYT in tackling oxidative stress.

Conclusion

The understanding of the exact role of CYT in ameliorating detrimental effects of salinity on plants is still limited as the previously conducted studies on the metabolism of CYT under stressful conditions present contradictory results. The objective of the current investigation was to understand the ameliorating mechanisms adopted by CYT in managing salinity at the cellular level. CYT had a positive impact in tackling salinity and imparting tolerance to pea by maintaining K^+/Na^+ homeostasis. This significant trait of salinity tolerance had been achieved by reducing the plasma membrane depolarization. The reduced depolarized state regulates the voltage-gated K^+ outward rectifying channels (GORK) and checking the K^+ efflux. CYT has significant role in controlling Na^+/H^+ (SOS1) antiporter increasing Na^+ efflux. The results for tackling oxidative stress by CYT were not consistent and needed further investigation.

Chapter 5: Ameliorating effects of exogenous cytokinin application on root ionic relations in salinized barley plants

Abstract

To a large extent, salinity stress tolerance mechanisms are confined to the root's ability to selectively regulate cation (Na^+ and K^+) uptake and sequestration within different root tissues and intracellular compartments. Accordingly, ameliorative effects of cytokinin (CYT) was studied in barley roots subjected to varying levels of NaCl treatment (50 & 100 mM). CYT was able to elicit a positive effect on root biomass and maintain high K^+/ Na^+ ratio in roots by preventing K^+ loss and Na^+ accumulation under salt stress. CYT treatment of barley roots enhanced the SOS1-like Na^+ extruding activity and induced the negative values of their membrane potential (MP) under saline conditions. Pharmacological experiments showed that effect of TEA^+ blocking outward rectifying K^+ channels (GORK blocker) was more pronounced while Gd^{3+} (NSCC blocker) effect was relatively minor. This outcome suggested that CYT had made plant salt-tolerant by retaining K^+ which can be facilitated by better control of GORK (activated by depolarization) and ROS-activated channels (such as NSCC) mediated K^+ efflux. It is concluded that CYT improves salinity stress tolerance via two concurrent mechanisms which include enhanced Na^+ efflux rate due to activation of SOS1-like Na^+/H^+ exchangers and improved K^+ retention. The efflux of K^+ is regulated by CYT through activation of H^+-ATPase and better membrane potential maintenance ultimately preventing GORK-mediated K^+ loss. Experiment with barley roots also indicated that there is a possible evidence for CYT being able to desensitize GORK activation by ROS.

Introduction

Excessive water deficit due to abiotic stresses such as salinity and drought lowers the crop productivity. Salinity suppresses plant growth and productivity by inflicting both ionic as well as and osmotic stresses (Munns and Tester 2008). Increased concentration of salts in the soil makes roots incapable of drawing water due to reduced water potential, and the build-up of salt concentrations inside plant hampers several metabolic activities leading to toxicity (Tavakkoli et al., 2011). Salinity also modulates the root system architecture by inhibiting primary root growth due to a fall in cell division and elongation (West et al., 2004). A change

in the microenvironment of the root system leads to a disturbance in the intracellular ionic homeostasis (Na^+, Ca^{2+}, pH and K^+), effecting the membrane potential and a broad array of biochemical processes. The state of membrane potential (depolarized/hyperpolarized) depends on the activity of the H^+-ATPase present in the plasma membrane (Hirsch et al., 1998). Therefore, perturbation in H^+-ATPase activity due to abiotic stresses including salinity results in a change in the ionic profile of the cell and often correlates with salinity tolerance, specifically in barley (Chen et al., 2007).

The mechanisms adopted by plants surviving in saline conditions include a balance between influx and efflux of Na^+, either back into the apoplast across the plasma membrane or the tonoplast into the vacuole . The devised tolerance mechanisms like compartmentalization of ions in vacuoles are executed by Na^+ /H^+ antiporters (NHX1) and salt extrusion through antiporter (SOS1) present in the plasma membrane. The presence of Na^+/H^+ antiporter in the plasma membrane of plants is critical for their growth under high salinity as it removes toxic Na^+ from the cytoplasm. Salt stress increases the activities of plasma membrane H^+-ATPase and Na^+/H^+ antiporter (Zhang et al. 2006). SOS1 gene from Arabidopsis was the first plasma membrane Na^+/H^+ antiporter gene cloned in higher plants (Shi et al. 2000). The overexpression of SOS1 in transgenic plants significantly improved salt-tolerance in Arabidopsis, while mutant plants lacking SOS1 protein were extremely sensitive to salt stress (Zhu et al. 1998).

Plant pre-treatment with various chemicals and hormones has often been used to ameliorate salinity stress impact on plants (Morgan and Drew, 1997; Danquah et al., 2014; Achard et al., 2008). One of such hormones is cytokinin (CYT) which plays a significant role in several vital processes in plant growth and development (Werner and Schmulling, 2009; Kieber and Schaller, 2014). The cytokinin response to environmental stimuli including abiotic stresses has only been recently characterized (Arguesoet al., 2009; Ha et al., 2012).CYT binding input domains (CHKs) with histidine kinase (HKs) comprise a two component CYT signaling pathway. Osmotic stress has been observed to induce expression of CHKs in *Arabidopsis*-AHK2 and AHK3 indicating involvement of the CYT in stress response. Salinity has a cytotoxic effect in addition to the adverse effect on water potential (Xiaong& Zhu, 2002). Under salinity stress, AHK2 and AHK3 are stimulated, and it has been reported that *ahk2*, *ahk3* (single mutants) and *ahk2,3* double mutants were more tolerant to salt stress (Tran et al., 2007). Also, arr1arr12, a double mutant of response regulator protein of CYT was shown to possess an enhanced tolerance to the salt stress (Mason et al., 2010).

The involvement of CYT in the K^+ uptake and transport in plants has been reported (Green & Muir 1979; Alizadeet al., 1990) although no specific details are known, and reports are often controversial. Lechowski (1997) reported the exogenous application of CYT to increase the K^+ in guard cells of stomata. At the same time, in another study kinetin couldn't elicit the activation of K^+ inward rectifying channels (KAT1) in guard cells (Mori, Uozumi& Muto 2000). Kinetin is observed to enhance the K^+ levels in excised roots (Waisel, Neumann &Eshel 1965); on the other hand, several reports claim the inhibition of K^+ release into xylem by kinetin (Rains 1969; Hong & Sucoff 1976). The evidence of regulation by CYT (1,4-dihydropyridine) of the voltage-gated Ca^{2+} channel (Schumaker&Gizinski, 1993) in moss and K^+ outward rectifying channels (TEA sensitive) in root epidermis of barley were presented (Shabala et al., 2009). The studies by Shabala et al. (2009) demonstrated that the uptake of the K^+ is controlled indirectly by kinetin transported into the cells. The regulation of K^+ flux through competitive inhibition of the pyruvate kinase and hexokinase by CYT also presents a possible route (Tuli, Dilley and Wittwer 1964). The apical requirement signal for K^+ uptake is regulated by apoplastic CYT.

In the previous chapter, the significance of CYT in maintaining K^+ levels on NaCl treatment was demonstrated for pea mesophyll cell. It was suggested that CYT pre-treatment enhanced H^+-ATPase activity under saline conditions which helped in reducing membrane depolarisation and reduced the K^+ efflux via depolarization-activated KOR channels. In the current investigation, the influence of CYT on K^+ retention and the nature of K^+ transporters involved was investigated in barley roots.

Materials and methods

Plant materials and growth conditions

Barley seeds (variety Gairdner) were obtained from the Australian Winter Cereals Collection and multiplied in the field of TIA (Tasmanian Institute of Agriculture) facilities at Launceston, Tasmania. Seeds were surface sterilized with commercial bleach containing 0.1% (v/v) Triton for 10 min and thoroughly rinsed with distilled water before use. Seeds were placed between two layers of wet filter paper vertically and then rolled properly. The size of the paper roll was 23 X 26 cm and eight seedlings were placed in the upper part of it. Distilled water was poured into a 225 ml beaker of 8 cm diameter. Paper roll then placed into the beaker and was

immersed into the BSM to a depth of 3–4 cm, so the plant roots never reached the solution during the entire period (4 days) of their growth. Capillary forces in the filter paper ensured that the plant roots remained wet and well aerated. Beakers were placed under constant (24°C) conditions in the darkened growth cabinet for four days. Seedlings were grouped according to different treatments of salt and CYT. For CYT and salt treatments stock solution of CYT and salt added to beakers to achieve desired concentrations required for experiments.

Measurements of physiological parameters

Fresh weight of roots was measured immediately after the removal of seedlings from paper rolls by Mettler BB2440 Delta Range balance (Mettler-Toledo, Griefensee, Switzerland). To determine root K^+ and Na^+ content, 0.1 g of ground dry barley roots were digested in 5 mL 70% HNO_3 and 2 ml 30% H_2O_2 in a 120 ml Teflon digestion vessel in microwave digester (MDS-2000 microwave digestion system, CEM Corporation, Mattews, NC, USA) for 1 h. The digested solution was then transferred into 15 ml centrifuge tubes. The solution was then diluted with distilled water to get a final volume of 15 ml and then again centrifuged at 5000 g for 10 min at room temperature (Avanti J-301cenrifuge, Beckman Coulter, and Krefeld, Germany). Supernatant of 0.2 ml was diluted to make a final volume of 10 ml, and K^+ and Na^+ contents were then measured by using a Flame Photometer (PFP7, Jenway, Stone, UK).

Ion flux measurements

Barley seedlings were gently placed in a 10-ml Perspex measuring chamber containing Basic Salt Media (BSM) solution (1 mM NaCl; 0.5 mM KCl; 0.1 mM $CaCl_2$; pH 5.7 non-buffered). Roots were gently placed at the center of a Perspex sample holder and fixed horizontally by movable plastic crossbars within the chamber. Treatments of NaCl and chemicals were applied by pipetting the required volume from stock solutions according to the experiments. Flux measurements during the first minute after treatment were discarded and presented as a gap in the figures. Non- filamentous borosilicate glass capillaries (GC 150-10, Harvard Apparatus Ltd, Kent, UK) were used to make electrodes. Prepared and calibrated ion selective microelectrodes were placed in a line parallel to root axis with 40 µM distance between tips and root surface (mature zone) along with a maintained gap of 1-2 µM between microelectrodes tips. A computer-controlled stepper motor (MO61-CE08, Superior Electric, Bristol, CT, USA) moved electrodes in a slow (6-s cycle,40 µM amplitude) square wave cycle

between two positions M1 and M2 from the seedling roots. The CHART software (Shabala et al. 1997) recorded the potential difference between two positions.

Membrane potential measurements

The root of barley seedlings was immobilized in a 10-ml Perspex measuring chamber containing BSM solution. Microelectrodes with a tip of nearly 0.5µm was filled with 1mM KCL and mounted in MIFE. A microelectrode was impaled into the epidermal cells of the mature root zone by a manually-operated micromanipulator (MMT-5, Narishige, Tokyo, Japan). CHART software was used to monitor membrane potential while impalement and membrane potentials were measured for at least 30 seconds in every experiment to achieve steady readings. The membrane potential values of eight individual seedlings were averaged for every treatment combination.

Pharmacology

TEACl (a putative K^+ channel blocker) and $GdCl_3$ (NSCCs blocker) were used to modify the activity of selected root plasma membrane transporters. These inhibitors were mixed with the basic solution (0.2 Mm KCl, 0.1 mM $CaCl_2$) to achieve their final concentration 20 mM TEA^+ and 100 µM Gd^{3+} respectively. After 1h pretreatment in the appropriate inhibitor, transient ion flux responses were measured.

Statistical Analysis

ANOVA program was used to analyze data statistically, and significance difference was compared at 5 % probability level using Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) software. Microsoft Excel, v 2010 was used to make graphs, and error bars in the graph represents standard error of six replications per treatment. Means sharing a common letter in the column are not significantly different at 5% probability. Error bars represents S.E.

Results

The treatment of CYT was given to the barley roots before subjecting them to the salinity (50 & 100 mM NaCl) treatment to investigate the physiological and ionic changes at

the cellular level. As the efficacy of CYT on plant physiological responses to salinity showed a clear doze-dependency (see the previous chapter of the thesis); an experiment was performed with varying concentrations of CYT (5, 10, 20, 50, 100 µM) to find the optimal concentration which could counteract detrimental effect of salinity on barley roots. The decrease in the barley root biomass was proportional to the rise in the severity of salinity. The positive results on the biomass of salt (50 & 100 mM NaCl) treated roots were only obtained with 5 µM CYT treatment. The enhancement in root weight was also observed in NaCl control samples due to root growth effect of CYT (Fig 1). The effect of salinity (50 & 100 mM NaCl) treatment and its mitigation by optimized CYT (i.e. 5 µM) was further observed with increase in the root biomass ($P < 0.05$) (Fig 2.1 & 2.2).

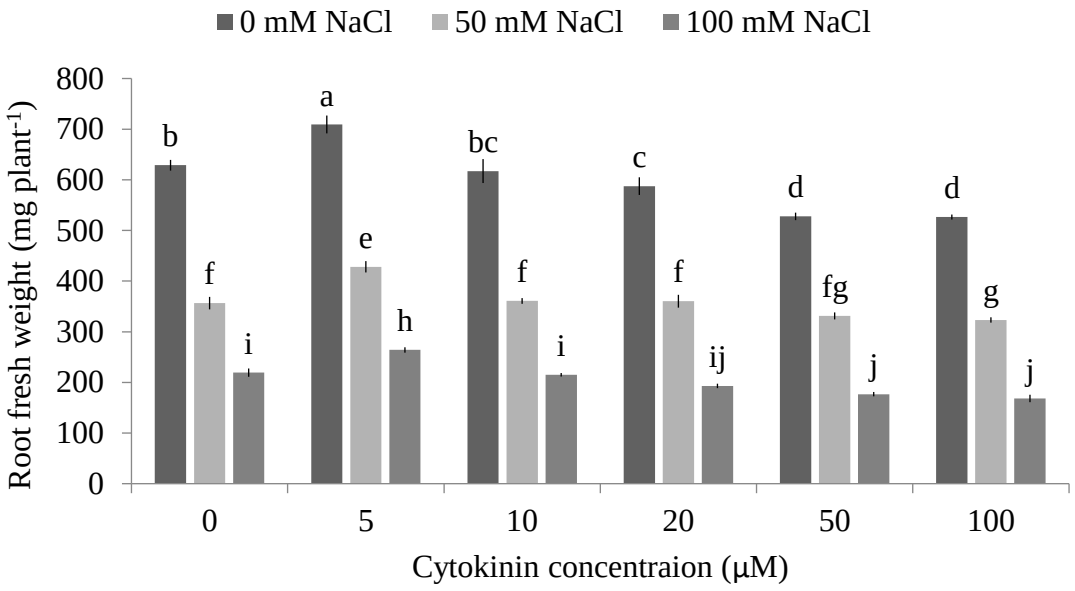

Figure 1-: Effect of cytokinins on the fresh weight of barley (cv Gairdner) roots grown under various levels of salinity (NaCl). To find optimum CYT concentration various concentrations of CYT have been added to the growth media, and plants were grown under saline conditions for three days until measurements were taken. Means ± SE ($n = 6$). Data with the different low case letters is statistically different at $P < 0.05$

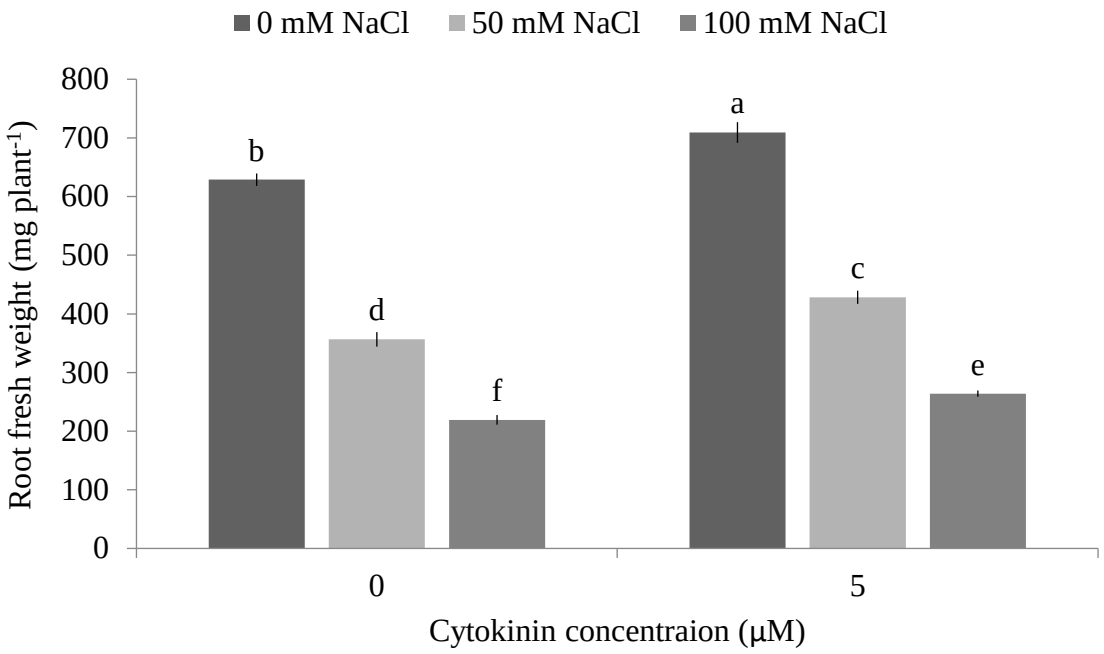

Figure 2.1-: Effect of cytokinins on the fresh weight of barley (cv Gairdner) roots grown under various levels of salinity (NaCl). An appropriate concentration of CYT have been added to the growth media, and plants were grown under saline conditions for three days until measurements were taken. Means ± SE (*n* = 6). Data with the different low case letters is statistically different at $P < 0.05$.

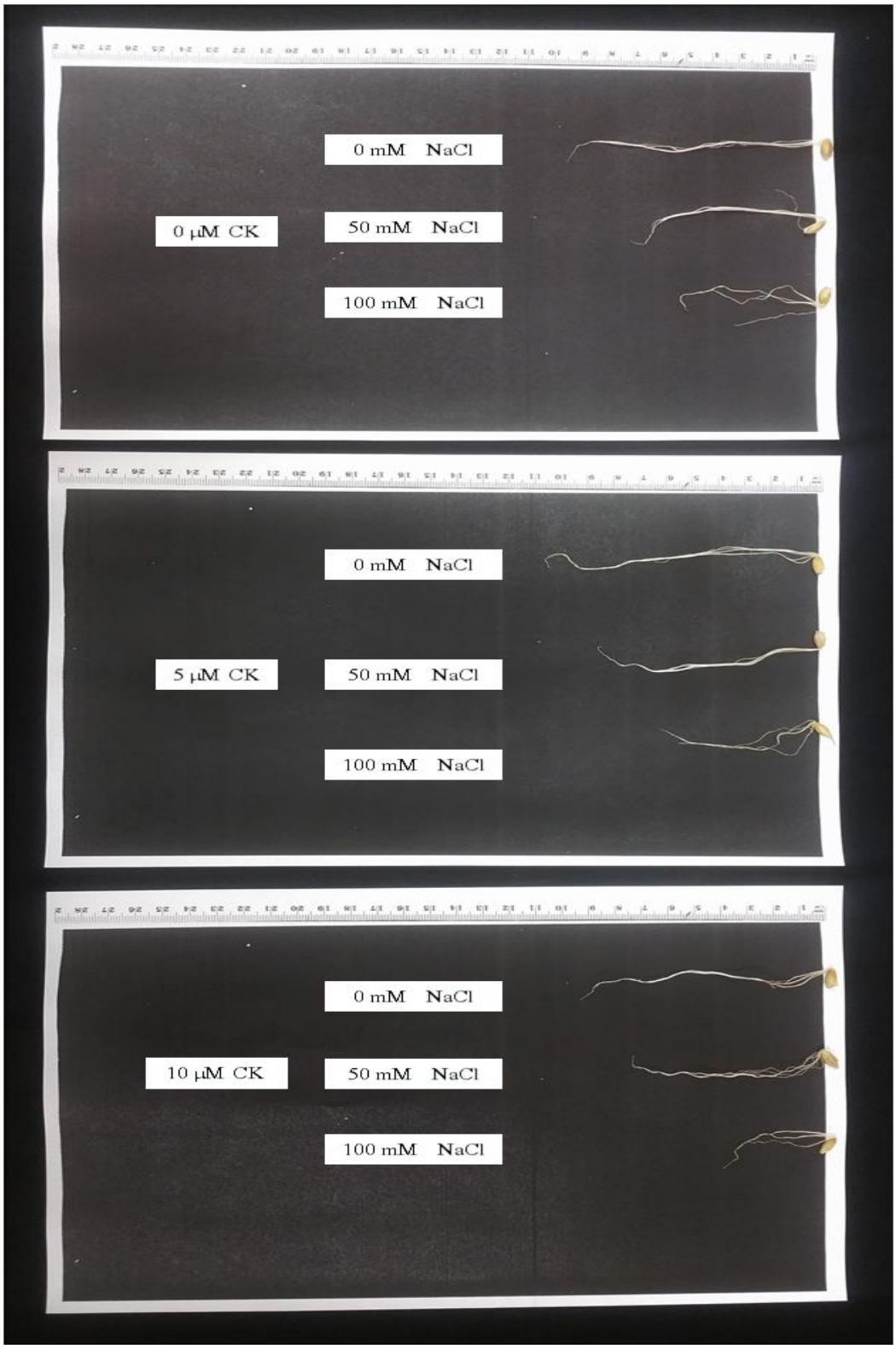

Figure 2.2-: Effect of cytokinins on the length of barley (cv Gairdner) roots grown under various levels of salinity (NaCl). An appropriate concentration of CYT have been added to the growth media, and plants were grown under saline conditions for three days until measurements & images were taken. Means ± SE (n = 6).

Salinity stress reduces plant K^+ content, and retention of K^+ in the cytosol in barley roots has been reported as a significant trait conferring salt-tolerance in this species (Chen et al. 2005, 2007). Here, salinity (NaCl) treatment led to a reduction in the root K^+ content (Fig 3.1). The application of 5 µM of CYT to the roots reduced the K^+ loss in roots (20% in 50 mM NaCl and 10% in 100 mM NaCl). The increase in salinity is coupled with a rise in the tissue Na^+ levels due to up-regulated Na^+ uptake. As the severity of salinity escalates the increase in Na^+ uptake through the barley roots as observed in Fig 3.2, the lesser Na^+ accumulation ($P< 0.005$) is seen in the test samples under salinity (50 & 100 mM NaCl) treatment upon the application of CYT (5 µM).

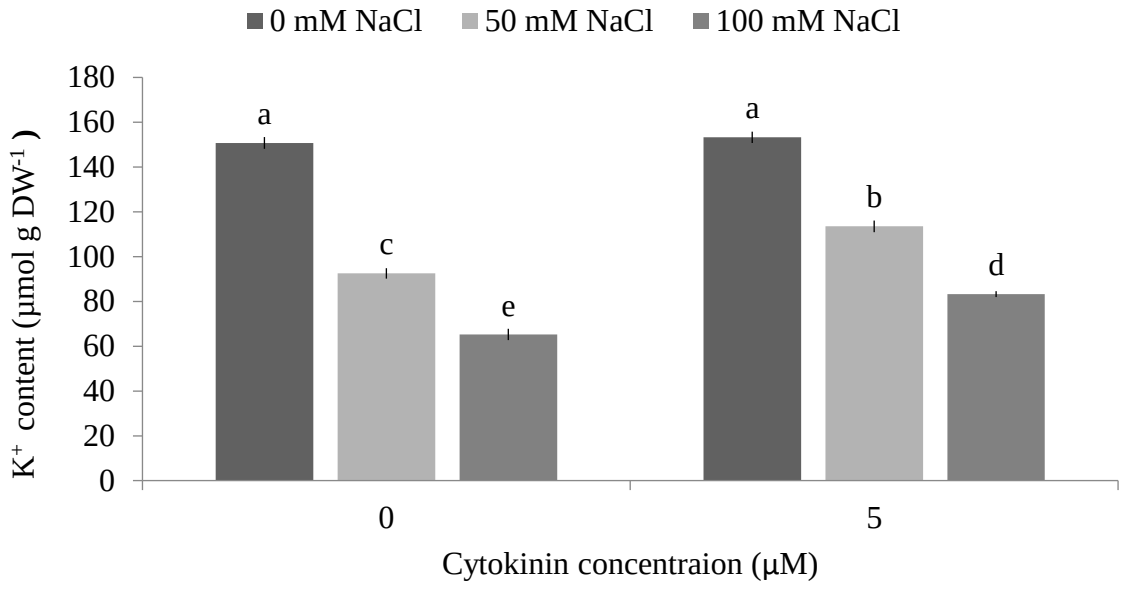

Figure 3.1-: Effect of cytokinins on the K^+ content of barley (cv Gairdner) roots grown under various levels of salinity (NaCl). An appropriate concentration of CYT has been added to the growth media, and plants were grown under saline conditions for three days until measurements were taken. Means ± SE (n = 6). Data with the different low case letters is statistically different at $P < 0.05$.

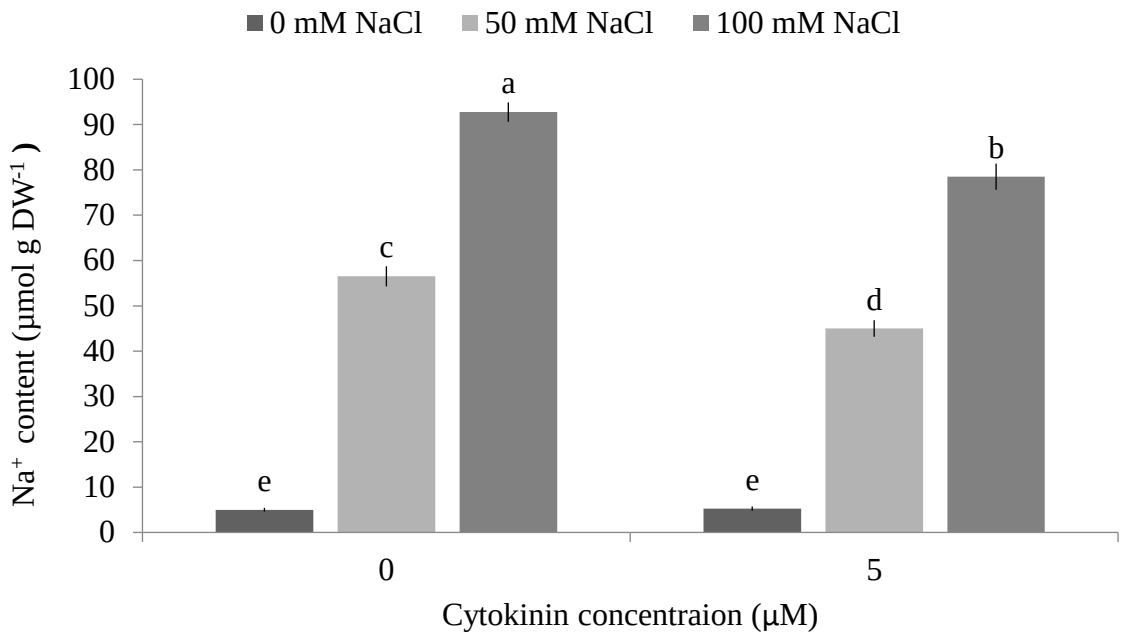

Figure 3.2-: Effect of cytokinins on the Na^+ content of barley (cv Gairdner) roots grown under various levels of salinity (NaCl). An appropriate concentration of CYT has been added to the growth media, and plants were grown under saline conditions for three days until measurements were taken. Means ± SE (n = 6). Data with the different low case letters are statistically different at $P < 0.05$.

The MIFE technique was employed to assess the combined effect of 100 mM NaCl treatment and 5 µM CYT on Na^+, K^+, H^+ & Ca^{2+} steady state flux response. The salinity has been a cause of immediate changes in net Na^+, K^+, H^+ and Ca^{2+} flux in barley root's mature zone. Acute 100 mM NaCl treatment resulted in a massive and prolonged net Na^+ uptake (Fig 4.1). Root pre-treatment with 5 µM CYT has shifted Na^+ flux towards net efflux (black circles in Fig 4.1). The exogenous application of the CYT also attenuated NaCl-induced efflux of K^+ (Fig 4.2). The efflux rate reached steady state after 10 mins. No clear trends have emerged for Ca^{2+} efflux upon NaCl stress (100 mM) on barley roots comparing control and CYT treated samples (Fig. 4.3). H^+ efflux was enhanced in both treatments but stronger in CYT-treated roots (Fig 4.4).

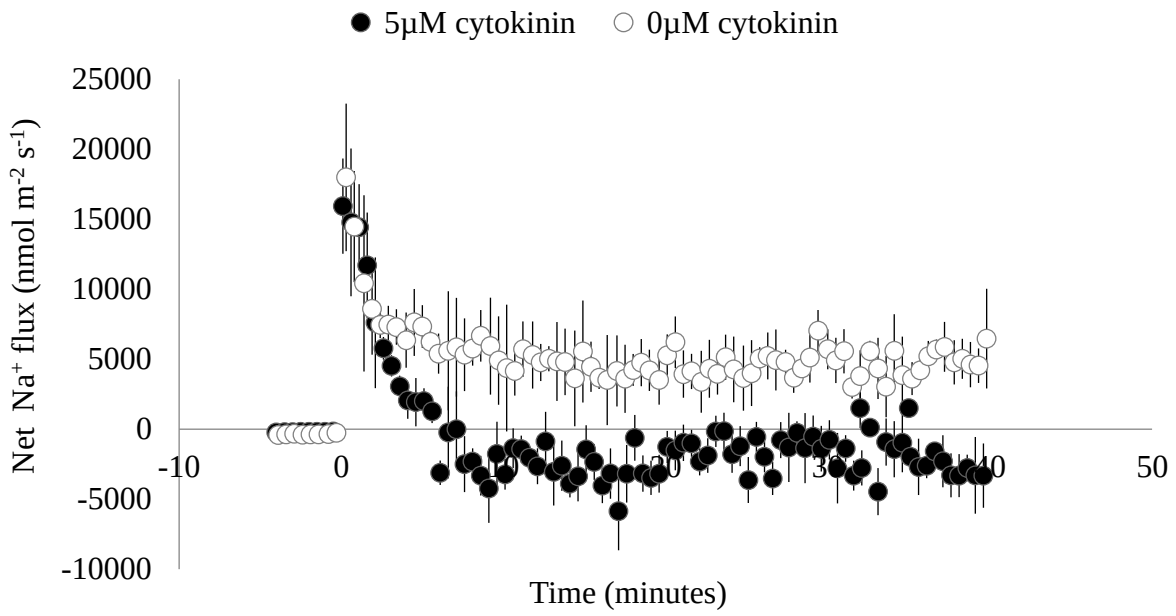

Figure 4.1-: Net Na^+ fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and in the presence of 5 µM CYT for three days in response to 100 mM NaCl treatment. The sign convention is efflux negative. Means ± SE (n = 6).

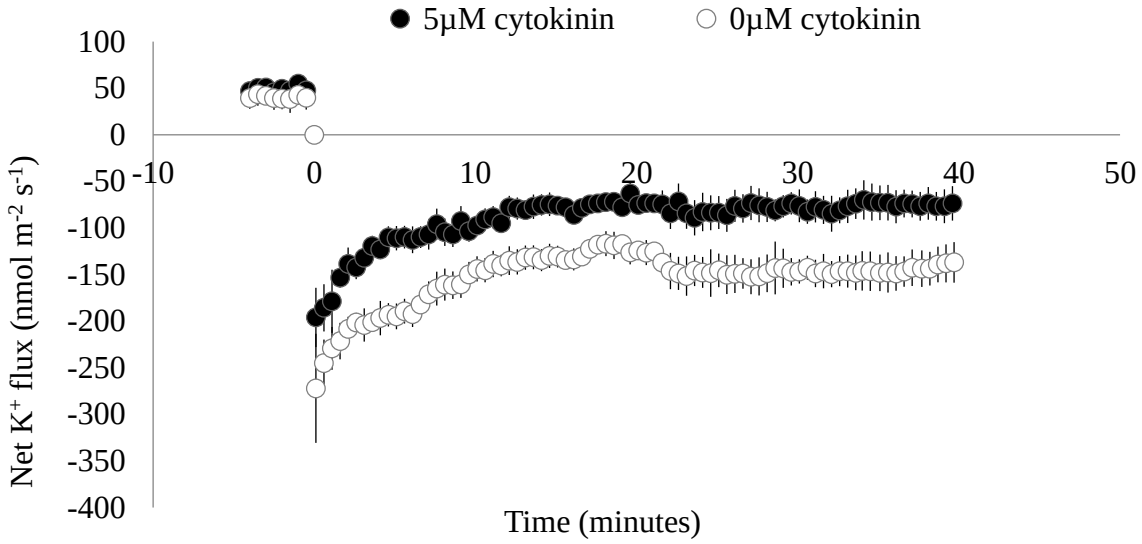

Figure 4.2-: Net K^+ fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and in the presence of 5 µM CYT for three days in response to 100 mM NaCl treatment. The sign convention is efflux negative. Means ± SE (n = 6).

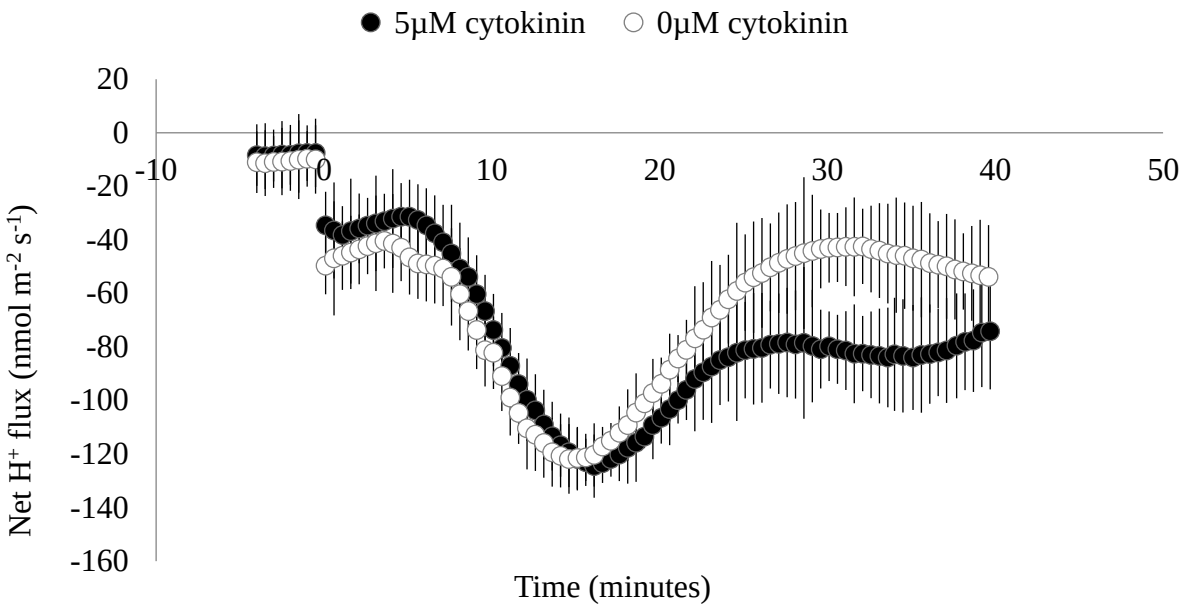

Figure 4.3-: Net H^+ fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and in the presence of 5 µM CYT for three days in response to 100 mM NaCl treatment. The sign convention is efflux negative. Means ± SE (n = 6).

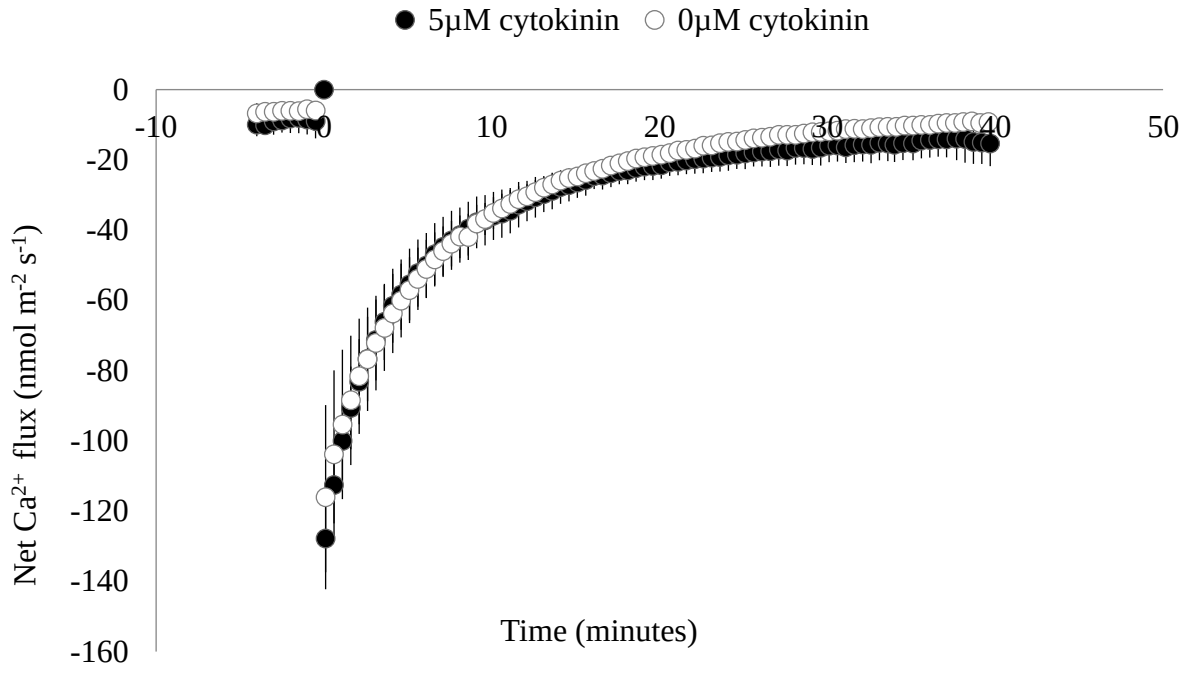

Figure 4.4-: Net Ca^{2+} fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and in the presence of 5 µM CYT for three days in response to 100 mM NaCl treatment. The sign convention is efflux negative. Means ± SE (n = 6).

The so-called "recovery protocol" was employed to quantify the functional activity of SOS1 (Na^+/H^+ antiporter) present in the plasma membrane (see Cuin et al 2011 for details). In brief, roots have been treated with NaCl for the time long enough to accumulate substantial amounts of Na^+ and induce the activity of SOS1-like Na^+/H^+ plasma membrane exchangers. Roots are then transferred into Na^+ free solution, and the measured steady Na^+ efflux is indicative of the functional activity of SOS1 transporter (as shown by Cuin et al. 2011 using Arabidopsis transport mutants). Here, Na^+ exclusion ability of barley root increased significantly ($P<0.05$) on treatment with CYT (5 µM) in comparison to control (Fig 5), indicating possible elicitation of SOS1 activity in leading to the extrusion of Na^+ from the cytosol.

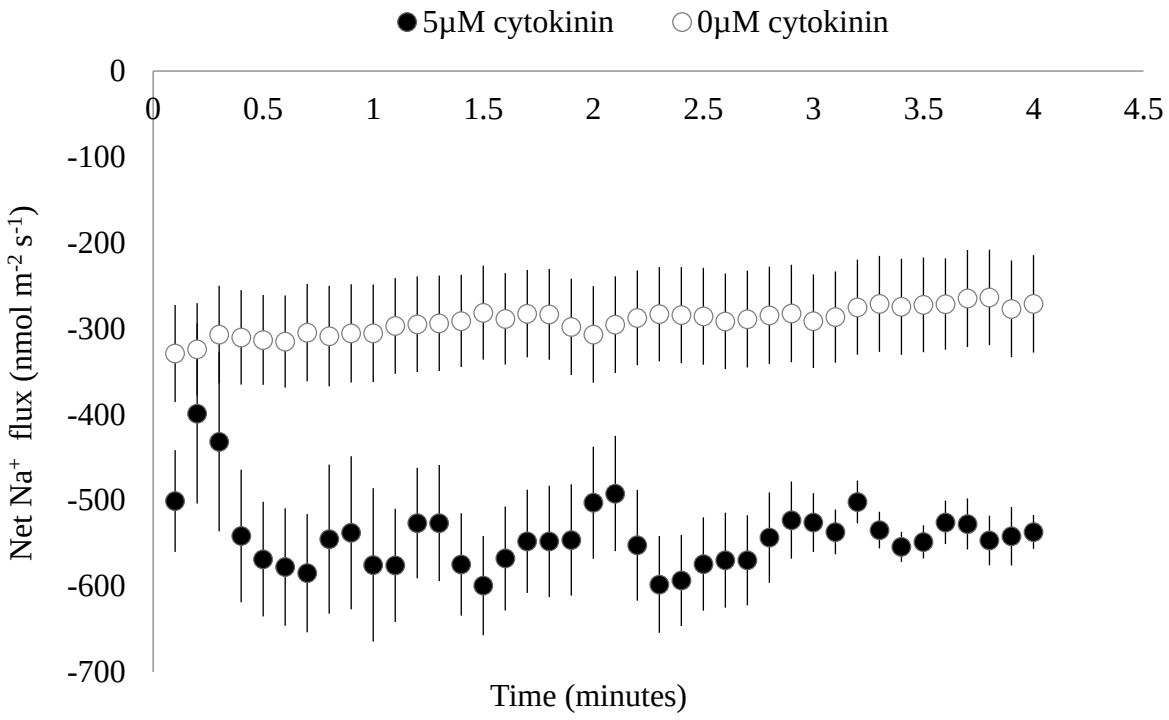

Figure 5-: Estimation of SOS1-like activity in barley roots in so-called "recovery experiments". Net Na^+ fluxes were measured from mature zone of barley (cv Gairdner) roots grown in the absence (control) and presence of 5 µM CYT after receiving 12 h of 100 mM NaCl treatment. Before measurements roots were washed in $CaCl_2$ and transferred in to Na^+ free BSM solution. The sign convention is efflux negative. Means ± SE (n = 6).

Combined effect of NaCl (50 mM) & CYT (5µM) pre-treatment (3 days) on the membrane potential of barley roots were tested (Fig. 6). The application of CYT shifted the resting potential towards more negative value in comparison to the control. This could be achieved by enhanced H^+-ATPase activity under salt stress which helped in reducing membrane depolarization. The repolarization of plasma membrane ultimately restricts the K^+ efflux via depolarization-activated KOR channels. The two channel blockers, TEA^+ (K^+-selective channel blocker) and $GdCl_3$ (non-specific cation channel blocker) were used in pharmacological experiments. Consistent with previous results significant steady K^+ efflux resulted after applying 100 mM NaCl. The treatment with 20 mM TEA^+ significantly (~ 90%) reduced the K^+ loss from roots of barley. The results suggest that the main contribution towards K^+ loss in response to NaCl are TEA^+ sensitive K^+ efflux channels (GORK). The effect of 100 µM Gd^{3+} treatment (a known blocker of non-selective cation channels, NSCC) on K^+ flux was minor indicating no effect of CYT on NSCC (Fig 7).

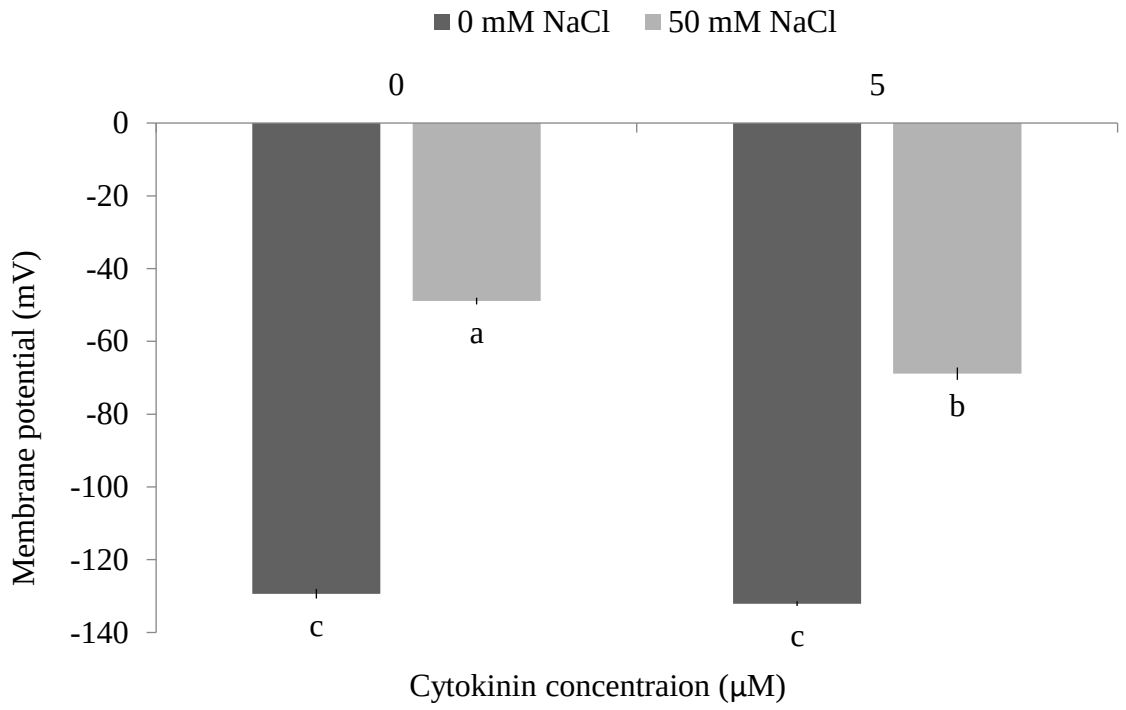

Figure 6-: Membrane potential measured from epidermal root cells of 4-d old barley seedlings grown in the absence (control) and presence of 5 µM CYT for three days in response to 100 mM NaCl treatment. Seedlings were treated with 0 & 50 mM NaCl for three days prior to measurement. Before measurements plants were washed in $CaCl_2$ and transferred in to Na^+ free BSM solution. Means ± SE (n = 6)

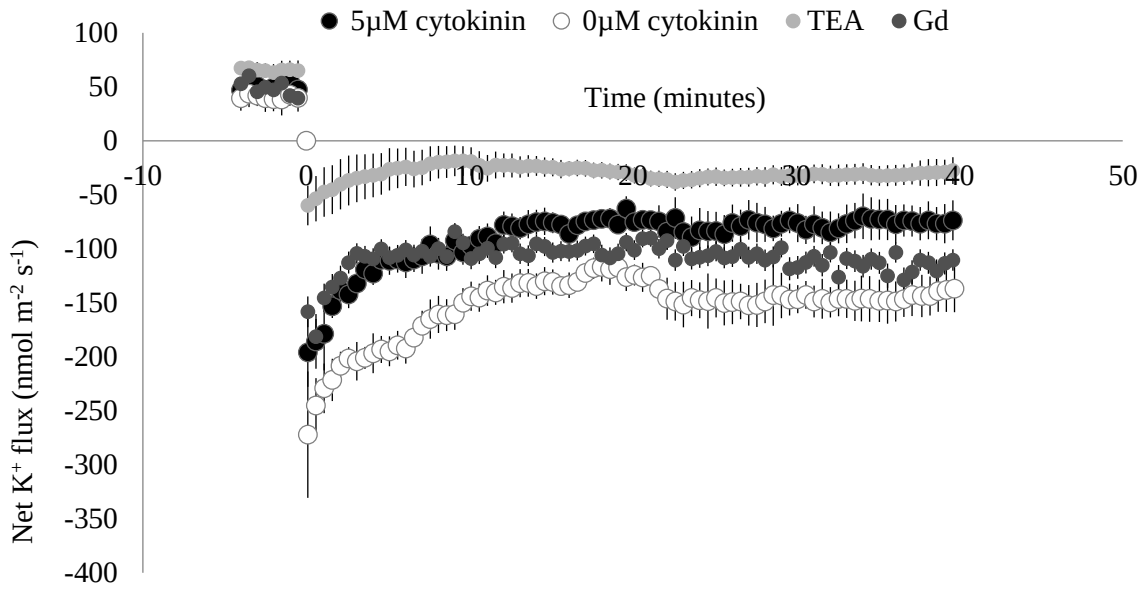

Figure 7-: Effect of ion channel blockers on NaCl-induced net K^+ fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and presence of 5 µM CYT. Either 20 mM TEA^+ or 100 µM Gd^{3+} were added to the bath medium one hour prior to applying 100 mM NaCl treatment. Means ± SE (n = 6).

Salinity stress is associated with increased ROS production (Mittler et al., 2010; Bose et al., 2014) that in turn activate K^+ permeable efflux channels (Demidchik et al., 2003, 2010). Can the ameliorating effect of CYT be related to mitigation of this ROS-induced activation? To answer this question, we looked at the effect of 3 days CYT treatment onto barley root subjected to hydroxyl stress-generating copper ascorbate (Cu/A) treatment. The hydroxyl ions generated from the application of Cu/A (0.3/1 mM) mix led to a massive K^+ efflux from the mature zone of barley roots (Fig. 8.1). The efflux of K^+ due to Cu/A (0.3/1 mM) mix treatment was not instant, but it reached peak values after 10 mins. The amelioration effect of CYT on K^+ retention was only visible after reaching the peak value. The generation of hydroxyl ions by Cu/A (0.3/1 mM) mix treatment increased the influx of the $Ca^{2+}$and H^+ in the mature zone of barley roots. The transient increase in influx rate of the Ca^{2+} reached peak in 10 mins of measurement (Fig 8.3). The same flux pattern was observed for H^+ which started to decrease after reaching peak value (Fig 8.2). The application of CYT had no significant positive effect on the influx response of H^+ and Ca^{2+} as induced by Cu/A (0.3/1 mM) mix treatment.

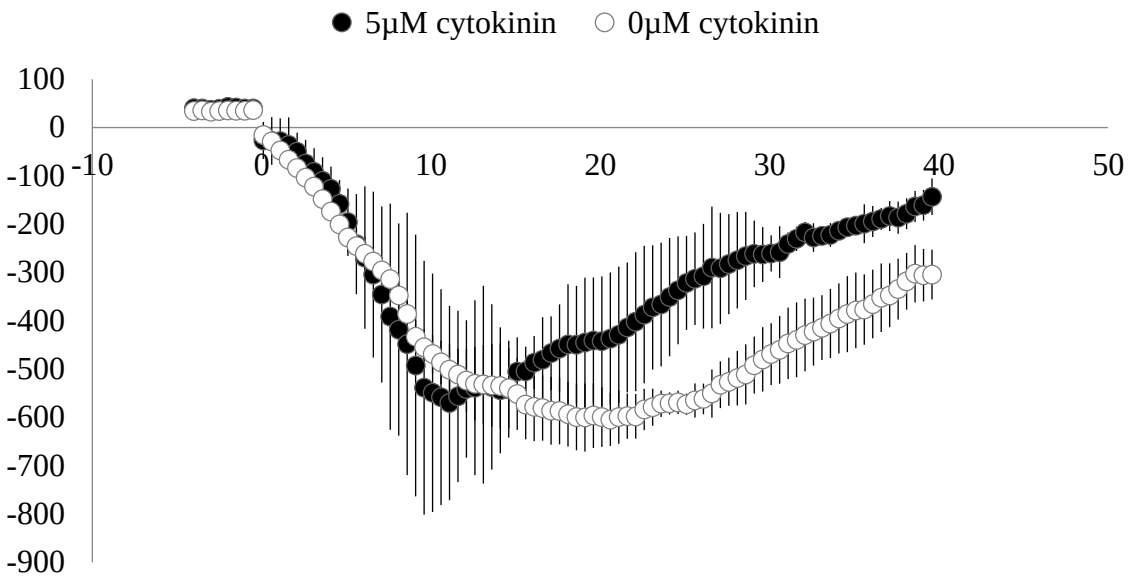

Figure 8.1-: Hydroxyl radical-induced net K^+ fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and 5 µM CYT (3 days) treatment. Hydroxyl radical-generating Cu/A (0.3/1 mM) mix was added at time zero. Means ± SE (*n* =6).

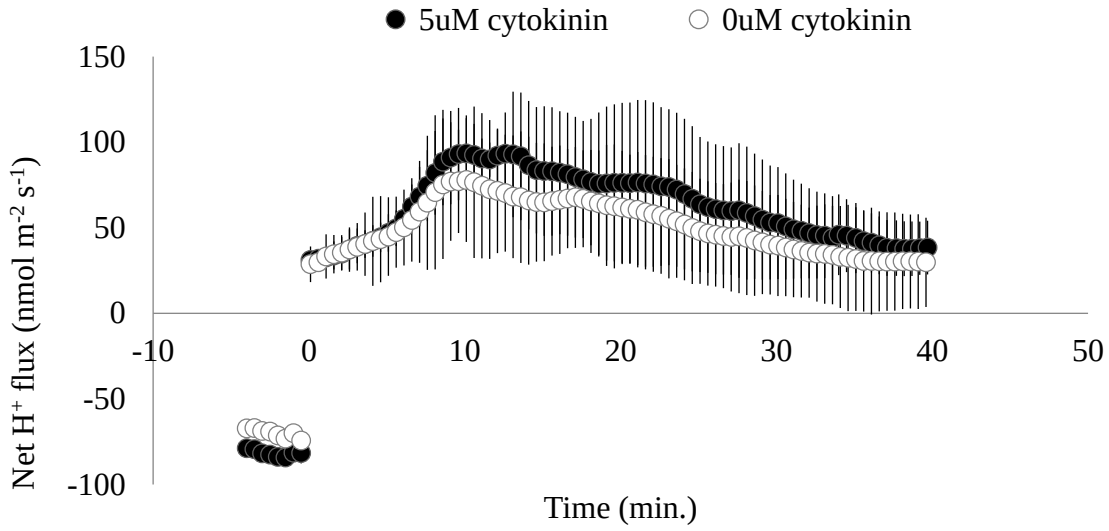

Figure 8.2-: Hydroxyl radical-induced net H^+ fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and presence of 5 µM CYT (3 days) treatment. Hydroxyl radical-generating Cu/A (0.3/1 mM) mix was added at time zero. Means ± SE (*n* = 6).

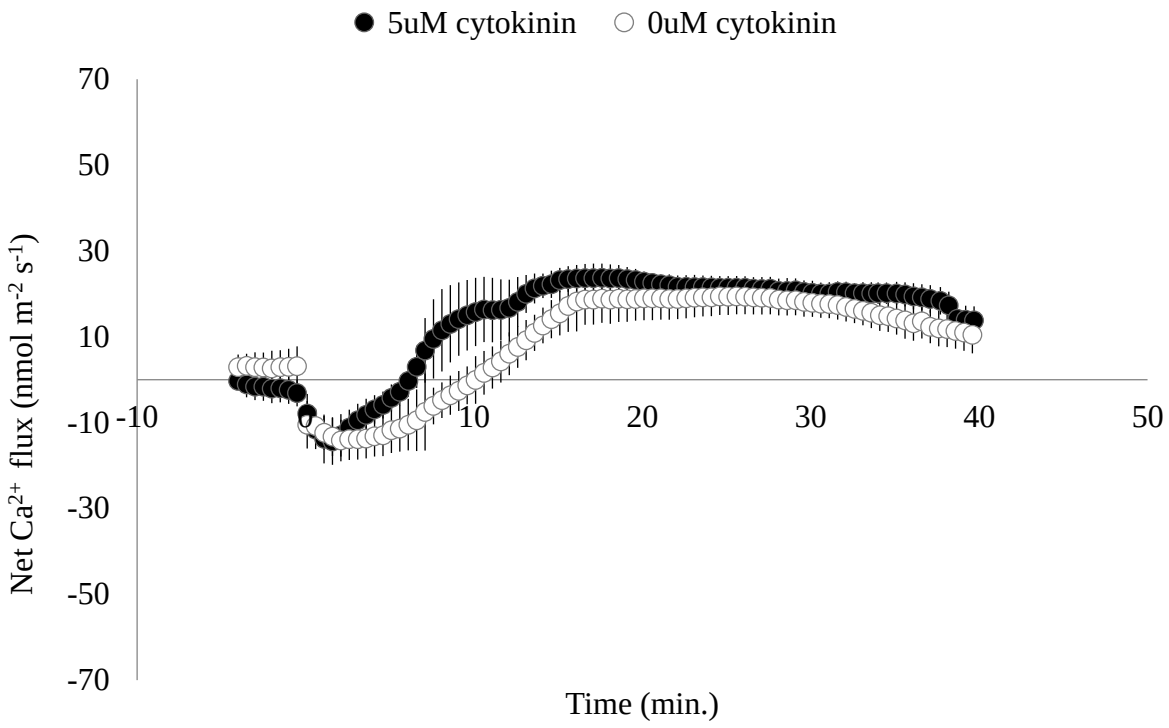

Figure 8.3-: Hydroxyl radical-induced net Ca^{2+} fluxes measured from mature zone of 4-d old barley (cv Gairdner) roots grown in the absence (control) and presence of 5 μM CYT (3 days) treatment. Hydroxyl radical-generating Cu/A (0.3/1 mM) mix was added at time zero. Means ± SE (*n* = 6).

Discussion

The purpose of the current investigation was to elucidate stress tolerance mechanisms confined to the root such as the ability to selectively regulate cation (Na^+ and K^+) uptake and sequestration in intracellular compartments under salinity. NaCl treatments (50 mM & 100 mM) decreased root fresh weight & potassium content while increasing the contents of Na^+.The significant reduction was observed in the biomass yield in terms of fresh weight of barley roots exposed to a long term salinity stress (3 days) during early development. The decrease in fresh and dry weight was inversely proportional to the salt concentration applied. This outcome shares similarity with that of Arabidopsis exposed to salinity for 2 weeks (Bose et al., 2013). The imbalance created in the ionic relations within the plant tissues has been attributed to the

growth and biomass reduction under salinity stress. The dose-dependency effect of CYT was experimentally evaluated with varying concentration of CYT (Fig 1). The concentration of 5 µM CYT was obtained as an optimal concentration which had significant amelioration effect on barley roots against salinity.

Three days NaCl treatment (50 mM & 100 mM) of barley seedlings increased the root Na^+ content and reduced the K^+ content (Fig 3.1 & 3.2). This observation was in agreement with the previous investigation on Na^+ accumulation causing salt sensitivity in rice (Yeo and Flowers 1986). High salt concentration in soil hamper the water uptake capacity of plant and influx of Na^+ by the roots impairs the metabolic processes (Flowers & Yeo 1995). The positive effect of foliar application of CYT on plants inflicted with salinity stress has been attributed partly to the regulation of K^+ transport (Green & Muir 1979) as observed in the present study. CYT treatment prevented NaCl-induce K^+ loss from the root, and K^+ retention in barley root is one the key attributes that confers salinity stress tolerance in this species.

The salinity has been a cause of immediate changes in the kinetics of net Na^+, K^+, H^+ and Ca^{2+} flux in barley roots. The presence of Na^+ in cytosol alters the ion homeostasis by inducing the exit of Ca^{2+}, H^+ along with K^+ from mesophyll (Shabala 2000). This calls for the need to monitor the ionic profile of the root tissue to establish the ameliorating effect of CYT in tackling ionic toxicity.

The pre-treatment of 100 mM NaCl before application of the CYT (5 µM) to the roots led to an influx of Na^+ through non-selective cation channels (NSCC) which are voltage independent and transport Na^+ preferentially (Maathuis and Amtmann 1999). The application of CYT lowered Na^+ accumulation in root tissues and even led to net Na^+ efflux from the root. The low levels of Na^+ in the cytosol can be maintained either by the limited entry or the efficient efflux of the ion from the cytosol. CYT was shown to induce the extrusion of Na^+ by stimulating Na^+/ H^+ antiporter (SOS1) located in the plasma membrane as established by results of recovery protocol. The proton motive force is created by the H^+-ATPase pumps for the secondary active transport (Dietz *et al*. 2001). The negative membrane potential at root is maintained by the H^+ pumps which are major consumers of ATP (Teakle*et al*. 2013). H^+ efflux down-regulate the GORK channels and simultaneously it provides the driving force for Na^+/H^+ exchanger (SOS) at plasma membrane removing excess of Na^+ in the cytoplasm (Chen et al., 2007).

The depolarization of the membrane potential under salinity is due to the excess entry of Na^+ into the root tissue through NSCC (Spalding et al.,1992; Demidchik and Tester, 2002). The excess entry results in an increased K^+ efflux through depolarization-activated outward-rectifying KOR channels and weakly voltage-dependent NSCC channels (Shabala, 2006). The net flux of K^+ and H^+ is a positive energy-saving strategy in salt-tolerant varieties. The membrane potential is restored with the increase in H^+ -ATPase activity closing GORK-like channels (K^+ outward rectifying channels) (Shabala and Cuin 2008). It has been observed that stimulation of H^+- ATPase down-regulate the GORK channels and simultaneously it provides the driving force for Na^+/H^+ exchanger (SOS) located in plasma membrane removing excess of Na^+ from the cytosol (Chen et al., 2007). CYT treatment had an influence on the efflux of H^+ only after 20 mins of measurements. We then measured MP of roots and showed that CYT-treated roots have a more negative MP values under saline conditions. CYT had more positive impact towards the restoration of membrane potential in the barley roots disturbed by salt stress (Fig 7).

With the rise in Na^+ concentration in the medium accumulation and influx of Ca^{2+} gets restricted (Lazof and Bernstein 1999). This restriction in the Ca^{2+} uptake by roots and further leads to a deficiency of Ca^{2+} in the whole plant of *Aloe vera* (Jin et al. 2007). In the current investigation, salinity interfered with Ca^{2+} in barley roots however CYT had shown no significant effect ($P< 0.005$) to enhance the Ca^{2+} levels.

One of the notable traits of salt-tolerance is the maintenance of high cytosolic K^+/Na^+ ratio. CYT treatment has been found to tackle the disturbance in ion homeostasis due to salinity by influencing K^+ uptake and Na^+ loss from the cytosol. To confirm the involvement of the particular channels in this pharmacological experiments were conducted with two channel blockers: (i) TEA (voltage-gated K^+ channel blocker) (Maathuis et al., 1997; Shabala et al., 2006) and (ii) $GdCl_3$ (non-specific cation channel blocker). The pretreatment with 100 mM NaCl 1 hour before measurement resulted in steady K^+ flux. This induced K^+ flux was checked with the application of TEA suggesting K^+ efflux channels sensitive to TEA^+ are the main contributors towards NaCl-induced K^+ loss in barley roots. The blocker of non-selective cation channels Gd^{3+} had a minor impact on K^+ flux. This observation leads to the conclusion that CYT affects the K^+ flux by GORK channels (voltage gated channels) and has comparatively less impact on the NSCC (voltage-independent channels).

The balance of ROS generation and scavenging gets altered under saline conditions leading to toxic accumulation of ROS in the cell (Mittler 2002; Apel and Hirt 2004). Several types of ROS are produced in stressed plants; H_2O_2 and hydroxyl radicals are arguably the most essential. Both types of ROS are known to disturb cell ionic homeostasis affecting the Ca^{2+} uptake and K^+ loss in Arabidopsis (Demidchik et al., 2003, 2007; Ordonez et al., 2012). In this study, H_2O_2 treatment also triggered net Ca^{2+} uptake and K^+ loss in barley roots, but CYT has no effect on these fluxes. Thus, it can be concluded that H_2O_2 signalling pathway and regulation of ion channels by peroxide is not affected by CYT.

On the contrary, K^+ extrusion from the mature zone of barley root caused by the exogenous application of hydroxyl radical-generating copper/ascorbate mix was reduced in roots treated with CYT (Fig 8.1). In both CYT-treated and control roots K^+ efflux reached its peak value after 10 mins developing gradually and afterwards showing recovery. After that, the K^+ loss was less in the CYT-treated root. It is known that Cu/A can directly activate GORK channels, as shown by Demidchik et al., (2010) in direct patch-clamp experiments. The positive influence of CYT on GORK channels has already been established through pharmacological results. Thus, experiments with Cu/A treatment on barley roots indicated that there is a potential evidence for CYT being able to desensitise GORK in response to ROS. To best of our knowledge this a first study to establish the role of CYT in retaining K^+ under salt and oxidative stress in barley roots.

Conclusion

The current investigation shows that exogenous application of CYT on a mature zone of barley enhances the salt-tolerance trait by maintaining a high K^+/Na^+ ratio. According to the suggested model based on current experiments-: CYT increases H^+- ATPase activity (as seen by the increased net MIFE H^+ efflux) hyperpolarizing the plasma membrane. This change in the membrane potential reduces salt-stress activation of voltage-gated K^+ outward rectifying channels (GORK) reducing K^+ loss. At the same time, increase H^+-ATPase activity “fuels” SOS1 Na^+/H^+ exchangers, reducing the Na^+ load in root cytosol. Also, it appears that CYT may desensitize sensitivity of some Na^+ and K^+ -permeable channels to ROS.

Chapter 6: General discussion

Developing strategies to counter growing concerns about decreasing agricultural land and loss of yield due to saline stress has become a top priority for growers and plant breeders. Due to the genetic and physiological complexity of the mechanisms of tolerance to salt stress, very few breeding efforts are becoming successful (Zhu 2000, Ashraf & Akram 2009). Most attempts to solve the problem by genetic manipulation of a few selected genes did not produce significant improvements in stress tolerance of culture salts under field conditions (Flowers 2004). Several studies suggested that salinity tolerance could be induced in plants by seed or foliar treatments with various phytohormones (Krishnamurthy 1991, Flowers 2004). Phytohormones treatment of plants conferred salt tolerance and an effect of improved growth and productivity (Farooq et al., 2009). In the current thesis nitric oxide (signalling molecule) & cytokinin (phytohormone) have been extensively investigated by monitoring their effect on physiological processes of salt-tolerant barley and salt-sensitive peas under salinity (NaCl) treatment.

The current study attempted to explain-:

1) In chapter 3, the ameliorative effect of nitric oxide donor (SNP) on salinity leaf tissue tolerance in barley and pea species has been investigated. The aim was accomplished through evaluation of combined effect of salt stress and foliar application of SNP (100 µM) on the plants by measurement of various physiological parameters under both *in vitro* and *in vivo* conditions. The result obtained from *in vivo* experiments done on whole plants was very different to the results obtained in *in vitro* experiments.

2) In chapter 4, the effect of CYT mitigation of oxidative stress in pea mesophyll under salinity was elucidated. The application of 20 µM CYT had a positive impact on physiological features such as CO_2 assimilation, photochemical efficiency, and chlorophyll content ($P< 0.005$). Laboratory studies using non-invasive MIFE ion flux measuring technique showed that Na^+ exclusion ability of pea mesophyll increased significantly ($P<0.05$) in CYT (20 µM) pre-treated plants as compared to that of non-treated, presumably by induction of SOS1 Na^+/H^+ exchanger. It is shown that CYT provided a protective effect by minimizing the efflux of K^+ induced by H_2O_2.

3) In chapter 5, the ameliorative effects of CYT (5 µM) were studied in barley roots subjected to varying levels of NaCl treatment (50 & 100 mM). CYT was able to elicit a positive effect on root biomass and maintain high K^+/ Na^+ ratio in roots by preventing K^+ loss and Na^+ accumulation under salt stress. CYT treatment of barley roots enhanced the SOS1-like Na^+ extruding activity and increased the negative values of their membrane potential (MP) on NaCl treatment. Maintaining a negative value of MP is an essential condition to prevent K^+ efflux through GORK channels. Pharmacological experiments showed that effect of TEA^+ (GORK blocker) was more pronounced while Gd^{3+} (NSCC blocker) effect was relatively minor in K^+ leakage under saline conditions.

The table summarizing new findings related to CYT and NO in this thesis are as following-:

Table 1: Comparative study of the earlier published observations and new observations obtained from the experimental work performed in this thesis employing NO and CYT in amelioration of salinity on physiological and cellular processes in barley and pea plant.

	Published work	**This study**
a)	Nitric oxide deficiency accelerates chlorophyll breakdown in Arabidopsis (Liu & Guo, 2013). NO retards the onset of chlorophyll degradation in broccoli (*Brassica oleracea*) florets (Eum et al.,2009) degradation in soybean (Glycine max) cotyledons (Jasid et al., 2009)	Positive effect of NO donor- SNP was prominent ($P< 0.05$) on the chlorophyll content in excised leaf segments of barley and pea but failed to work for *in planta* conditions. The most likely explanation is that there is a certain "concentration window" within which the effect is observed.
b)	Previously, a positive effect of exogenous application of NO was reported on photochemical efficiency of cucumber under salinity (Fan et al., 2007).	Photochemical efficiency of both barley cultivars and pea leaf discs exposed to salt stress was enhanced.
c)	The treatment of spinach plants with a low concentration of NO gas (ambient atmosphere with NO gas (200 nLL^{-1}) significantly increased the shoot biomass (Jin et al., 2009). In pea & wheat a foliar application of NO had a dual behavior. Low micromolar concentrations produced an increase in the rate of leaf expansion, whereas no promotive effect occurred at higher concentrations	SNP (100 µM) application did not show any ameliorative effects on biomass yield, chlorophyll content, CO_2 assimilation in either cultivar of barley (Gairdner & CM 72) and pea plants grown in a glass house, subjected to NaCl treatment (200 & 400 mM for barley, 50 & 100 mM for pea).

	(Leshem, 1996; Tian & Lei, 2006). NO significantly improved the gas exchange attributes and CO_2 assimilation in salt-stressed rice cultivars (Habib et al., 2013) and in salt-stressed tomato plants (Wu et al., 2010).	
d)	Exogenous application of NO improves plant K^+ contents while decreasing Na^+ concentration, thereby maintaining the K^+/ Na^+ ratio in plants under salt stress (Zheng et al., 2009). The application of NO resulted in an increase of net efflux rate of Na^+ in *Avicennia marina* (Chen et al. 2010). The application of NO donor SNP was found to up-regulate the expression of AKT1 in the roots of NaCl treated *Kandelia obovata*, suggesting the significance of NO in increasing K^+ uptake in salt-treated roots (Berthomieu et al, 2003).	NO foliar spray did not alter xylem and leaf sap concentrations of $Na^{+,}$ K^+ & Cl^- ions in glasshouse grown barley and pea.
e)	Salama and Awadalla (1987) have reported a significant increase in plant growth under salinity through the application of kinetin.	Application of 20 µM CYT substantially increased shoot fresh and dry weight of glass house grown pea plant treated with 50 & 100 mM NaCl.
f)	Exogenous application of CYT has been observed to counteract the water stress caused by salinity and alleviating its effect on the activity of the RUBISCO or PEP carboxylase, maintaining photo-efficiency of PS I and II as well as chlorophyll content (Metwally et al. 1997, Pandey et al. 2000).	Treatment of 20 µM spray of CYT has helped to recover the Fv/Fm ratio by approximately 12.5%, thereby preventing detrimental effects on leaf photochemistry of pea mesophyll. CYT treatment on the plants exposed to salinity increased the chlorophyll content.
g)	The positive effect of foliar application of CYT on plants inflicted with salinity stress has been suggested to be linked with regulation of K^+ transport (Green & Muir 1979).	The exogenous application of the CYT reduced the efflux rate of K^+ through GORK channels.
h)	No direct investigation on CYT effect on Na^+ transport across plant membranes was conducted	The recovery protocol measurement of Na^+ flux in the barley roots and pea mesophyll showed enhancement of the

		SOS1-mediated Na^+ exclusion ability under the influence of CYT as reported.

6.1 Effect of NO and CYT as foliar spray on physiological and photosynthetic efficiency of NaCl treated barley and pea

Taking into account of previous studies reporting the positive influence of NO & CYT on salt stressed plants, a comprehensive study of monitoring effect of NO & CYT foliar spray on the glasshouse grown barley and pea was undertaken. Pea and barley differed in their tolerance to salinity. After optimization, under *in vivo* conditions NO foliar spray on the plants of barley and pea showed that NO application was incapable in improving pea and barley growth under salt stress. This is reflected in the growth, CO_2 assimilation, chlorophyll content and photochemical efficiency of these plants under salt stress. However, outcomes of the *in vitro* experiment on whole plants were not similar to outcomes of the *in vivo* experiments. The variation in the efficacy of NO as foliar spray in comparison to its direct application on excised leaf segment is suggested to be due to the (1) the fact that this molecule is sensitive to photodegradation (2) its short life span, and (3) the existence of a "concentration window" within which NO operates. The effect of CYT in management of NaCl induced salinity stress was assessed in terms of biomass yield, photochemical efficiency and CO_2 assimilation. The experiments were performed comparing the biomass yield of the pea seedlings under medium (50 mM) and high (100 mM) NaCl concentration with varying doses of CYT application. The extent of reduction in the fresh and dry weights of the plants recorded significant amelioration effect under the influence of CYT (20 μM & 50 μM). Interestingly, better results were obtained with 20 μM CYT with a lesser extent of biomass reduction in comparison to control.

6.2 Ionic profile of pea and barley on exogenous application of NO & CYT under salt stress

The Na^+ exclusion from transpiration stream is the primary response of plants to salinity and can impart salt tolerance (Gorham et al., 1990; Shi et al., 2002). However, exposure to long-term salinity stress causes substantial leakage of K^+ from the cytosol (Shabala, 2000), inhibition of photosynthesis (Shabala et al., 2010), increase in ROS production (Miller et al. 2010) and reduction in stomatal aperture (Brugnoli and Lauteri 1991). The concentration

of Na^+ in the leaf sap was directly proportional to the concentration of NaCl applied to the plants and the quantitative trend appeared almost similar in both the cultivars of barley (cv CM72 and Gairdner). The leaf ion concentration estimates have indicated a reduction in Na^+ and Cl^- concentrations and a significant improvement in the concentration of K^+ with application of 20 µM CYT. This finding suggests the prevention of accumulation of Na^+ and Cl^- synergize with retention of K^+ under the influence of the applied dose of CYT. However, a foliar spray treament of SNP (100µM) could not bring a positive impact on the leaf sap K^+/Na^+ ratio under salinity (NaCl) treatment.

6.3 Maintenance of K^+/ Na^+ homeostasis by CYT

The current study suggests that the prevention of Na^+ accumulation under salt stress is synergistic with the retention of K^+ under the influence of the applied dose of CYT. The presence of Na^+ in the cytosol altered the ion homeostasis and led to efflux of Ca^{2+}, H^+ along with K^+ from mesophyll (Shabala 2000). The effect of CYT on the flux of Ca^{2+}, H^+ flux was non-significant. The experimental results clearly display the effect of CYT in maintaining high K^+/ Na^+ ratio in both pea and barley plants. To confirm the participation of the particular channels (if any), pharmacological experiments were conducted with two channel blockers; 1) TEA- (K^+ channel blocker) 2) $GdCl_3$ (non-specific cation channel blocker). This finding suggests that K^+ efflux channels sensitive to TEA^+ are the main contributors towards NaCl-induced K^+ loss in barley roots. $GdCl_3$ (non-specific cation channel blocker) had a minor impact on K^+ flux which implies that CYT contributes to the K^+ retention under salt stress by controlling the K^+ flux by GORK channels (voltage gated channels).

6.4 Management of ROS by CYT

The exogenous application of ROS Cu/A (0.3/1 mM) mix resulted in significant K^+ extrusion from mature zone of barley roots. The K^+ efflux response was not instantaneous as it reached peak value after 10 minutes developing gradually in barley roots and 15 minutes in pea mesophyll. The amelioration effect of CYT in barley roots on K^+ retention was only visible after reaching the peak value. Exogenous application of ROS H_2O_2 (10mM)) on pea mesophyll induced a significant K^+ efflux. Pre-treatment of pea mesophyll with CYT (20µM) showed lower efflux ($P<0.05$) in comparison to the control when H_2O_2 (10 mM) was applied. The positive effect of CYT in barley roots on K^+ retention was only visible after 10 minutes.

From the whole study, it is concluded that CYT increase salt tolerance in pea mesophyll and barley roots. The better K^+ retention as a result of CYT application under salinity (NaCl) treatment seems to be due to its influence on the GORK channel to counteract the oxidative stress. CYT had more positive impact towards the restoration of membrane potential disturbed by salt stress in the pea mesophyll and barley roots. The membrane potential is restored with the increase in H^+-ATPase activity closing GORK-like channels (K^+ outward rectifying channels). The enhanced H^+- ATPase activity also provides the driving force for Na^+/H^+ exchanger (SOS) located in plasma membrane removing excess of Na^+ from cytosol (Chen et al 2007).

Based on above observations, the tentative models of CYT operation in plant root and mesophyll tissues are proposed (Figs 6.1 and 6.2)

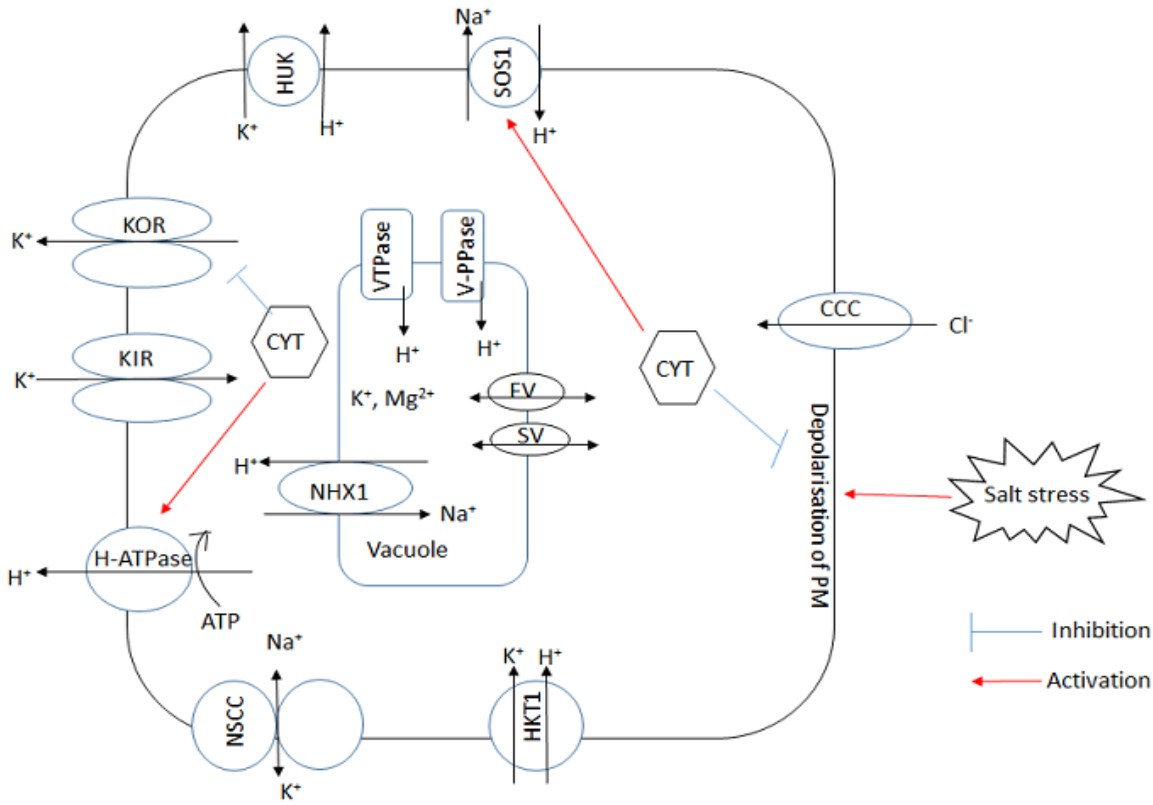

Figure 6.1-: Effect of CYT on the ion transporters present in barley root cell under salt stress. KOR- Outward rectifying K^+ channels; HKT1- high affinity K^+ transporter; NSCC- non selective cation channels; KIR- inward rectifying K^+ channels; SOS1 – Na^+/H^+ antiporter (plasma membrane); NHX 1- Na^+/H^+ antiporter (tonoplast); SV- slow-activating vacuolar channels; FV- fast-activating vacuolar channels; HUK/HKT- K^+ transporters

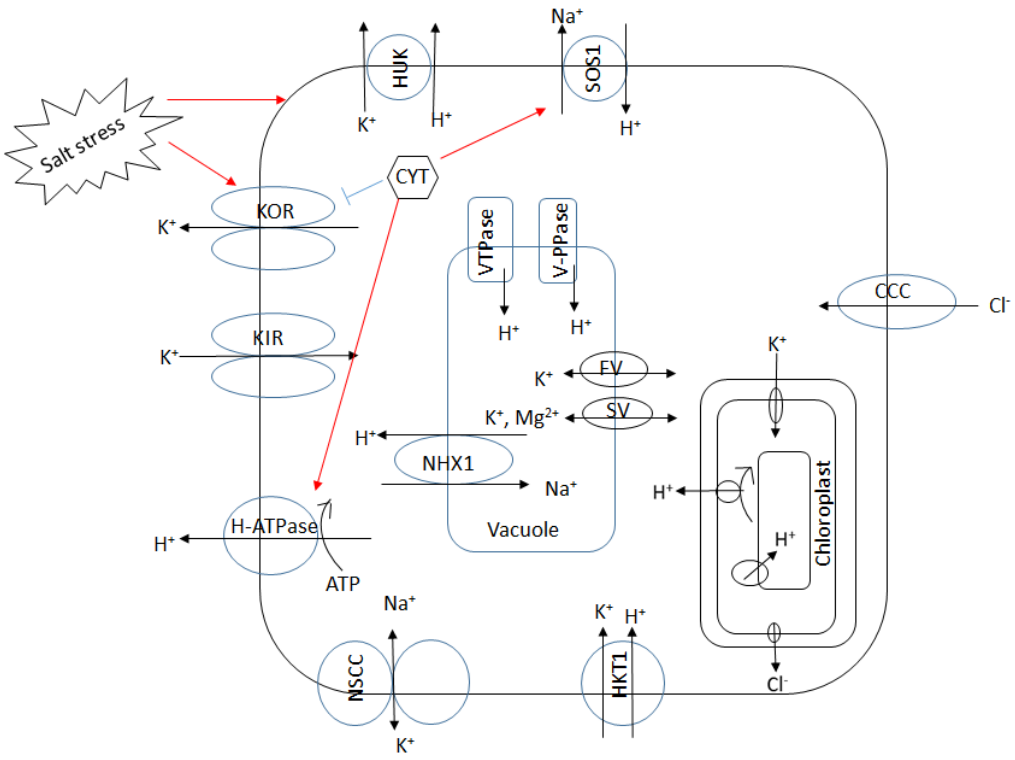

Figure 6.2-: Effect of CYT on ion transporters present in the pea mesophyll cell.

HUK/HKT- K^+ transporters; KOR- Outward rectifying K^+ channels; HKT1- high affinity K^+ transporter; NSCC- non selective cation channels; KIR- inward rectifying K^+ channels; SOS1 – Na^+/H^+ antiporter (plasma membrane); NHX 1- Na^+/H^+ antiporter (tonoplast); SV- slow-activating vacuolar channels; FV- fast-activating vacuolar channels;

6.5 Conclusion

The present project demonstrates that application of SNP as foliar spray on the intact plants of barley and pea plants did not have significant beneficial effects against NaCl toxicity. In the two sets of experiments (*in vivo & in vitro*) results of *in vitro* experiments study

demonstrated a positive impact of NO in improving the chlorophyll content and photochemical efficiency of barley and pea leaves. However, outcomes of the *in vivo* experiment on whole plants were not at par with outcomes of the *in vitro* experiments. Uncertainties and challenges in ensuring efficient and uniform absorbance of SNP stray into the leaf tissue was most likely one of the main reasons negating its efficacy when applied to intact plants. Therefore, application of NO in the field for equipping crop plants with salt stress resistance needs further review. The established mechanisms which can be contributing to the positive effect in leaf discs of barley and pea are related to reduce ROS accumulation by activating antioxidant enzymes. By quantifying the hydrogen peroxide and superoxide levels using fluorescent dyes the role of NO in tackling oxidative stress could be ascertained. The future work requires measuring the steady state K^+ and Na^+ fluxes from the mesophyll of the leaf treated with NaCl with and without SNP application to validate the efficacy of NO treatment in excised leaf segments.

To the best of my knowledge, the role of CYT in mitigation of oxidative stress and ion toxicity in barley and pea plant has not been reported at cell-specific level. The new findings of the current work with CYT fill in the gap in knowledge of exact adaptive mechanisms induced by CYT and clear the ambiguity regarding the its impact (positive/negative) on the plant under stressful conditions. CYT had a positive impact in tackling salinity and imparting tolerance to pea mesophyll and mature zone of barley by maintaining high K^+/Na^+ ratio. CYT has significant role in controlling Na^+/H^+ (SOS1) antiporter increasing Na^+ efflux. The retention of K^+ by CYT due its influence on GORK channel is a significant attribute in counteracting oxidative stress and all this contributes to improved salinity tolerance in CYT treated barley roots and pea mesophyll. The identified traits of salt tolerance influenced by CYT can be introgressed into commercial plants by marker-assisted selection. Overexpression of CYT biosynthesis could be good option provide it should not imbalance the internal hormonal ratio in plants. It has been reported by several studies that increase in cytokinin synthesis would enhance the salt tolerance. For example in transgenic *Sinorhizobium* overexpressing *ipt* gene had improved tolerance to severe drought stress. Water deficit conditions were found to reduce the CYT levels in plants, but overexpression of isopentenyltransferase (IPT), an enzyme involved in CYT generation, alleviated this decrease, and hence enhanced stress tolerance (Havlová et al., 2008; Ghanem et al., 2008). Cytokinin has been established as suitable protectant against adverse effects of salinity *in vitro* and additional set of experiments comprising ameliorating effect on biomass yield, Fv/Fm, CO_2 assimilation,

chlorophyll content, ion flux response, and K^+/Na^+ concentration in xylem have to be conducted on greenhouse grown plants to validate its efficacy in field application. The current thesis also opens up the possibility of using cocktail comprising of several protective substances including CYT and NO against salinity after testing their interactive effect in the future in order to make it more realistic and consumer friendly. Salicylic acid is a potential option to mix with CYT and NO.

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